Thesis for the Master's degree in chemistry

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I: Investigation of the effect of column temperature in capillary reversed phase high performance liquid chromatography of proteins.

II: Fractionation and separation of basic plasma proteins using two dimensional liquid chromatography.

60 study points

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Preface:

This graduate study has been carried out at the University of Oslo, Department of Chemistry in the period of august 2004 to june 2005. My supervisors have been professor Elsa Lundanes, Professor Tyge Greibrokk, and PhD student Milaim Pepaj.
It is a two-part thesis, both parts concerning optimizations of methods for LC-separation of proteins. Part one describes a two-dimensional separation using conventional LC, while part two deals with column temperature regulation on capillary-LC systems.
Coming from the smaller Agder College and attending the University of Oslo after working a couple of years was a tough task. In the beginning, I felt totally lost. Fortunately, all the people I have been in contact with from the Department of Chemistry; students, teachers and staff have been very helpful. I would like to thank you all for being very nice and patient having to explain and show me everything.
A special thanks to:

My supervisors: Elsa Lundanes, Tyge Greibrokk, and Milaim Pepaj for an interesting project and brilliant guidance.

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The technical staff at the Department of Chemistry for being helpful, as good as all time available, and extremely patient.

Hanne Britt Johannesen at Ullevål University Hospital for allowing me to work and make some badly needed money during the study. You have been very flexible. I would also like to thank you for granting me a two-year leave while completing the study.

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Finally I would like to thank Heidi for all support and patience.

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Abstract part I:

Investigation of the effect of column temperature in capillary reversed phase high performance liquid chromatography of proteins

The effect of column temperature on separation of proteins in reversed phases capillary LC has been investigated in the range of 25ºC-125ºC. Five proteins have been used as models to study changes in retention time and recovery using a 150 x 0.3 mm PLRP-S column and a water/Acn mobile phase gradient. Generally, the retention time decreases as the temperature increases. No significant reduction in recovery was found below 100ºC.

Furthermore, the proteins were heated prior to injection to cause denaturation, and the effect of denaturation was measured. All model proteins but one showed reduced recovery after being heated to 75ºC and 100ºC for one hour. In no case could renaturation be observed after cooling the proteins for 24 hours. Instead, the recovery of some proteins tended to decrease even more after this "storage".

It has also been investigated how protein denaturation is influenced by the time period the proteins are exposed to high temperature (residence time). Increased column residence time did not result in more denaturation below 100ºC during a measurement period of 60 minutes. At 100ºC, a significant decrease in recovery was observed for most proteins when exceeding 30 minutes additional residence time on the column.

Additionally, separation of two and three proteins using temperature gradient as an alternative to mobile phase gradient has been investigated. Satisfying separation was accomplished using both mobile phase and temperature gradient without any significant difference in recovery between the modes.

Furthermore, the use of temperature to aid separation of complex mixtures has been investigated on a monolithical 60 x 0.180 mm column. A mobile phase gradient that almost separated a mixture of ten proteins at 30ºC was carried out under different temperature conditions, flow rates, and with or without a preheater to heat the mobile phase prior to the column to investigate if the separation could be improved. Complete separation of all the

proteins was not achieved, but the effect of temperature was clearly seen by comparing the chromatograms generated under different conditions.

Abstract part II:

Fractionation and separation of basic plasma proteins using two-dimensional liquid chromatography

In this study, a method for on-line two-dimensional separation of basic proteins in plasma was investigated. The proteins were separated according to their pI value using a strong anion exchange column. Selected fractions were further separated according to hydrophobicity using a PLRP column.

The on-line two-dimensional system failed. Large interfering peaks appeared during the second dimension. Mobile phase components and the trap columns were pointed out as the source of the interferences, but no strategy was developed to deal with the problem.

An alternative off-line system was developed. The method was tested once without providing satisfying separation and was rejected because it was a labour and time consuming method, involving several manual steps.

Part I:

Investigation of the effect of column temperature in reversed phase capillary liquid chromatography of proteins.

1. **Introduction:**

Liquid chromatography (LC) is an essential tool for separation, purification, and characterization of proteins. Of the many chromatographic principles, reversed phase has proven to be suitable for separation of many proteins [1-2]. Selectivity can easily be manipulated by changing the mobile phase. The recovery is generally good, also for proteins present at low abundance, and usually, relatively good repeatability and reproducibility are obtained.

Separation of macromolecules is a more difficult task than separation of smaller molecules. Regarding proteins and peptides, two factors due to the nature of the proteins contribute to the difficulties.

Firstly, the size and structure of the protein will complicate the retention process [3-7]. Proteins are built up of small residues known as amino acids, which are asymmetrically distributed along a non branched chain. The chain is folded and makes a three-dimensional structure, where only parts of the residues, generally the hydrophilic ones, are represented on the surface. The rest is buried inside the molecule. Only residues with access to the surface can interact with the stationary phase and contribute to the retention process. Various residues have different affinity for the stationary phase and steric limitations prevent all groups from interacting simultaneously. The mass transport within and between the phases is generally poor for macromolecules. The diffusion in and out of the stationary phase is a relatively slow process, which contributes to increased plate height.

A second problem arises from the unstable conformation of proteins [8-12]. First of all, the typical protein conformation is bendable and changes all the time. This internal flexibility is highly necessary for proteins to operate and provide their tasks sufficiently in living organisms. The slightest changes in the environment may cause internal changes to the structure. During such internal changes, the protein surface area usually remains intact. Due to steric limitations, the entire surface area can not usually interact with the stationary phase simultaneously, and a change in formation may cause the protein to interact differently with the stationary phase over time.

A rough three-dimensional structure is anyhow characteristic for a certain protein under normal conditions. The structure that the protein upholds under normal conditions is called the native state. It is not fully understood how a given amino acid sequence obtains this characteristic conformation, but it is believed that a major contribution to the folding is the repulsing forces between the aqueous environment and hydrophobic residues [8-9]. The result is a packed three-dimensional arrangement of hydrophobic groups buried inside the macromolecule and the hydrophilic groups represented on the surface. The peptide chain backbone is itself polar and the parts of the branch that are buried within the protein with the hydrophobic residues will adopt the most energetic favourable state by interacting with itself and make structures known as α-helixes and β-sheets, which represent the protein's "skeleton".

The energy required to rupture the native state is not very high, only about 0.4 KJ/mol amino acid [9]. When the native state collapses the protein unfolds and exposes buried residues to its environment. This is called protein denaturation. Neither the process nor the nature of denaturated proteins is very well understood. Under certain conditions some proteins may take up new conformations, while under other conditions it seems that the process results in more random structures [8-9, 13].

In reversed phase LC, temperature and uses of gradient elution can be used to improve efficiency, [14-15] and reduce retention time [16]. It has been suggested and experimentally confirmed that a change in column temperature affects the separation of proteins [4].
The transfer process between the phases is dependent upon the protein's affinity for the different phases. This can be described as a thermodynamic process where the free energy involved is expressed by the Van´t Hoff equation:

$$\ln k = -\Delta H/RT + \Delta S/R + \ln \Phi \quad (1)$$

k is retention factor, ΔH is change in enthalpy, ΔS is change in entropy, R is the gas constant, T is absolute temperature and Φ represents the fraction of mobile phase and stationary phase that fills the column.

In general, the effect of changing the temperature is greater for large molecules than for smaller ones [17]. Different protein species gain a different k value. A change in temperature

may affect the retention differently and may even change the retention order of the involved proteins. For proteins where k changes differently, temperature manipulation can be used to improve the separation of the proteins.

Temperature can on one hand be used to improve the chromatographic efficiency and selectivity, and allow faster and better separation of proteins. On the other hand, temperature can contribute to destabilize the proteins' native states and may cause protein denaturation [8-9]. When denaturation occurs, the proteins expose more hydrophobic groups that can strongly interact with the hydrophobic stationary phase and result in larger retention. In some cases, the proteins might even attach permanently to the stationary phase.

2. Aim of study:

The aim of the present study was to investigate the chromatographic behaviour of proteins on reversed phase columns in a capillary LC system, primarily to examine how the column temperature affects the separation. Five model proteins with different sizes and pI values were selected to investigate the behaviour of individual properties using a PLRP-S column. A mixture containing 10 different proteins was used to investigate the effect of temperature on separation of complex mixtures using a polystyrene based monolithical column.

3. Experimental:

3.1 Proteins:

All proteins (Table 1) were obtained as dried samples except apoferritin that was 25 mg/ml in 50% glycerol and 0.075 M NaCl. All proteins were purchased from Sigma-Aldrich (St.Louis, MO, USA).

Ten different proteins were used during this study. Out of these, five model proteins in two mixtures were selected for an investigation of the properties of individual proteins (Mix I and II), while a third mixture contained all ten proteins (Mix III). See table 1.

All proteins were dissolved in type I distilled water, except fibrinogen and hemoglobin, which were dissolved in 2 mM NaCl solution.

The mixtures were stored in portions of 1 ml in microcentrifuge plastic cups from BRAND (Wertheim, Germany) in a freezer (approx -18^{o}C). When in use, the solutions were stored in a refrigerator (approx +4$^{\circ C}$) for maximum 10 days before disposing.

Table 1: Proteins used in this study and their molecular mass and pI values. All molecular masses and pI values were obtained from Sigma-Aldrich.

			Consentration (mg/ml)		
	Molecular weight (Da)	**pI**	**Mix I**	**Mix II**	**Mix III**
Cytochrome-C (bovine heart)	12,327	10.25	0.5	-	0.05
Lysozyme (chicken egg)	14,300	11.0	0.5	-	0.05
Myoglobin (horse heart)	17,000	8.0	0.5	-	0.05
Albumin bovine (chicken egg)	66,000	4.7	-	0.5	0.50
Apoferritin (horse spleen)	443,000	4.0*	-	0.5	0.25
β-lactoglobulin-B	18,300	5.1	-	-	0.50
Fibrinogen	23,700	9.3	-	-	0.50**
Conalbumin (chicken egg)	~ 77,000	N/A	-	-	0.50
Hemoglobin (rabbit)	N/A	6.8	-	-	0.50**
Ovalbumin	44,300	5.54	-	-	0.50

** Not determined experimentally, approximated by Sigma-Aldrich.*

*** Dissolved in 2mM Nacl*

3.2 **Chemicals:**

All water used for mobile phases and preparation of samples was type I distilled water, obtained using Milli-Q gradient system (non-TOC) water purification generating 18MΩcm ultrapure water quality from Milllipore S. A. S. (Molsheim, France).
Acetonitrile (Acn) HPLC grade S quality was obtained from Rathburn Chemicals ltd. (Walkerburn, Scotland, UK)
Trifluoroacetic acid (TFA) UV spectroscopy quality > 99% was from Fluka Chemiclas. (Buchs, Switzerland)
NaCl >99.5% was from Sigma-Aldrich.

3.3 **Instrumentation and procedures:**

The instrumentation is schematically shown in Figure 1.
The capillary gradient pump was an Agilent 1000 series Capillary pump from Agilent Technologies. (Waldbronn, Germany). The mobile phases contained A; water added 0.1% TFA and B; Acn added 0.1 % TFA as in reservoir A. Eventual changes in mobile phase composition is described under the actual experiment. The flow rate was most of the time 10 µl/min and is specified under the actual experiment if different. Manual injection was performed with a valve-loop injector with 500 nl loop from Valco instruments (Houston, TX, USA). The columns were a PLRP-S column 15 cm x 0.3 mm i.d. with 1000 Å pore size and 5 µm particles from G&T SEPTECH AS (Oppegård, Norway) for experiments involving Mix I and II and a 6 cm x 0.180 mm i.d. poly(styrene)-co-divinylbenzene monolithic column [18] for experiments involving Mix III.

To generate the wanted column temperature, the column was placed inside a Polaratherm series 9000 oven from Selerity Technologies Inc (Salt Lake City, UT, USA). The oven was equipped with a preheater prior to the column to generate the temperature of choice for the mobile phase before entering the column. The preheater was connected directly to the injector port (outside the oven) and the column inlet (inside the oven) The preheater and the column were connected with a 1/16 x 0.005 inches (0.127 mm) stainless steel connection and fitted with a special graphite ferrule for high temperatures (delivered with oven). The preheater had a total length of 45 cm, with i.d. 0.005 inches. (0.127 mm) The oven system further included a mobile phase peltier cooling device to cool the mobile phase after the column and a CO_2 operated cooling system to control the oven temperature and rapidly cool the oven between the runs. The peltier cooling device was connected to the column outlet similar to the preheater, had the same dimensions, and a total length of 50 cm. All other connections were fused silica capillaries obtained from Polymicro Technologies Inc (Phonex, AZ, USA), and fittings were FS1.4 graphite ferrules obtained from Valco. The operating range for the oven was specified to be -20º to 200ºC with ±0.5ºC at constant temperature, and ±1ºC during temperature programs for increasing the temperature 1°C-30ºC/min up to 150ºC. The proteins were detected with either Spectra 100 variable UV/Vis wavelength detector from Spectra-Physics (Mountain View, CA, USA) (Mix I and Mix III) operated at 214 nm or Z-cell UV detector K-2600 UV detector with Z-cell from KNAUER (Berlin, Germany) operated at 280

nm (Mix II). Software for readout and calculations was Totalchrom Navigator 2003 from Perkin Elmer Inc (Wellesley, MA, USA).

All experiments were carried out on the same system. The column was placed inside an oven prior to the column, inside the oven, the mobile phase passed through a preheater to achieve the oven temperature (Mix I and II). In experiments involving Mix III the preheater was disconnected and replaced with a capillary 50 cm x 180 µm i.d. After the separation, the mobile phase was cooled to 25ºC before detection for experiments with Mix I and II. The outlet of the peltier was connected to a capillary (i.d. 100 µm, total length 45 cm) passing through the detector. The distance from the peltier outlet to the detector readout was 20 cm for Mix I and Mix II. In the experiments using Mix III the cooler was disconnected. The mobile phase was instead lead directly from the column outlet to the detector (outside the oven) via a capillary (50 µm i.d., total length 50cm). The distance from the column outlet to the detector readout was 24.5 cm. A CO_2 operated cryo cooling device assisted maintaining constant temperature by preventing the temperature to rise above the selected temperature and helped cooling the oven between the runs. The cryo cooling was not attached during the experiments with mixture III. A 2 cm x 20 µm restrictor was connected to the capillary outlet for all mixtures.

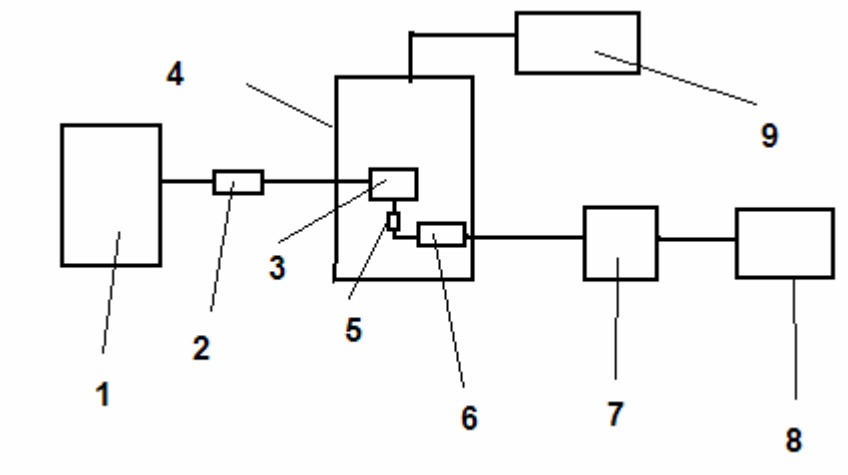


Figure 1: Capillary LC-UV instrumentation: 1. Gradient pump, 2. Manual valve injector, 3. Mobile phase preheater, 4 .Oven, 5. Column., 6. Mobile phase peltier cooling, 7. Detector, 8. Readout, 9. Cryo oven cooling.

3.4 **Calculations and data treatment:**

All experiments were carried out using three replicates. The mean value of the replicates was used as the result. The standard deviation was also calculated.
In cases where one sample was anomalous to the other two a 90% confidence interval Q-test was performed to possibly reject the result:

$$Q_n = \frac{|x_a - x_b|}{R} \quad (2)$$

R is the range of all data points, x_a is the suspected outlier, and x_b is the data point closest to x_a.

Critical value at 90% confidence interval and three samples is 0.94 [19].
Comparisons of peak areas were used to estimate the recovery. All data is presented relative to a reference which was usually the peak area obtained at room temperature. The reference is specified for the different experiments in the results and discussion part.
Due to the relatively large standard deviations within the sample replicates, a variance analysis of the data sets that were to be compared was carried out (ANOVA). Since the values can change both ways, a two tailed test was performed. For a 95% confidence interval and three replicates within each sample the critical value is 12.22 [20].

The retention factor in the Van't Hoff plot (results and discussion experiment 1) was calculated by the formula $k = (t_R - t_0)/t_0$. k is retention factor, t_0 is retention of a non retained component and t_R is retention time of the actual protein. t_0 was determined by injecting uracil, a compound expected to be unretained on a reversed phase column.

4. **Results and discussion:**

4.1 **The temperature effect on retention time and recovery of individual proteins:**

To investigate the properties of individual proteins (4.1-4. 4), a polystyrene based reversed phase column with relatively large pore size, 1000 Å was selected (see experimental). The

macroporous styrene/divinylbenzene material can stand high temperature over time without suffering from degradation (150ºC in water) and the large pore sizes are ideal for separation of a large span of large molecules (MW 5000-500,000 Da) [21].

Protein Mix I and II were chromatographed with the same solvent gradient and under the same conditions; only the column temperature was changed. Four temperatures were investigated, 25ºC (reference), 50ºC, 75ºC, and 100ºC. All data are presented relatively to the values achieved at 25ºC. The chromatograms are shown in figure 2 and 3 relative area and retention is shown in figures 4 and 5.

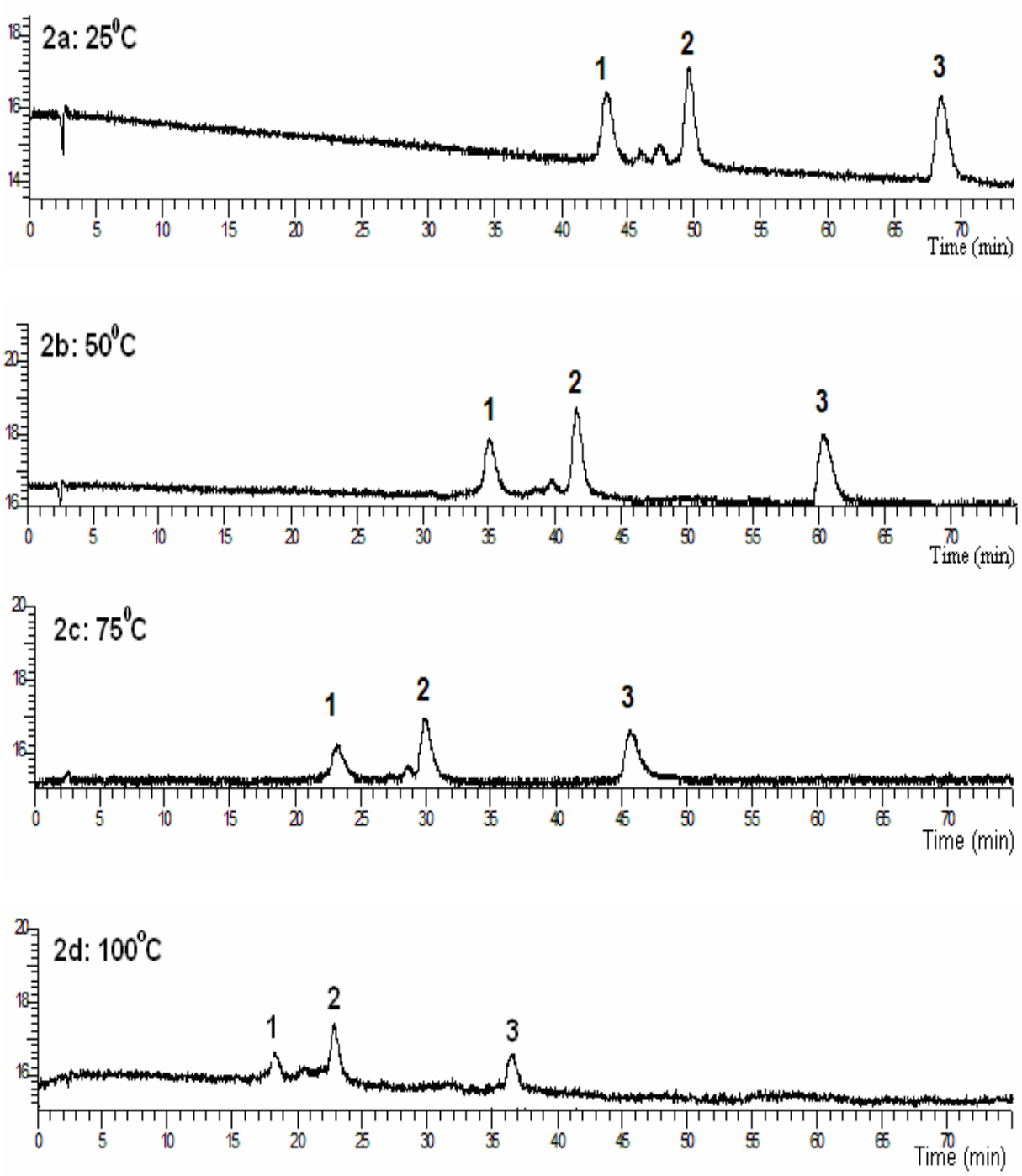


Figure 2 a-d: Chromatograms of Mix I; cytochrome-C (1), lysozyme (2), and myoglobin (3). The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size at 25ºC, 50ºC, 75ºC, and 100ºC. Mobile phase flow was 10 µl/min, 500 nl was injected and UV detection after separation was achieved at 25ºC at 214 nm. A gradient running from 20% -50% B in 60 minutes, followed by 50% -100% B in 25 minutes, and 100% B for 5 minutes was used. Mobile phase A was water + 0.1% TFA and mobile phase B was Acn + 0.1% TFA.

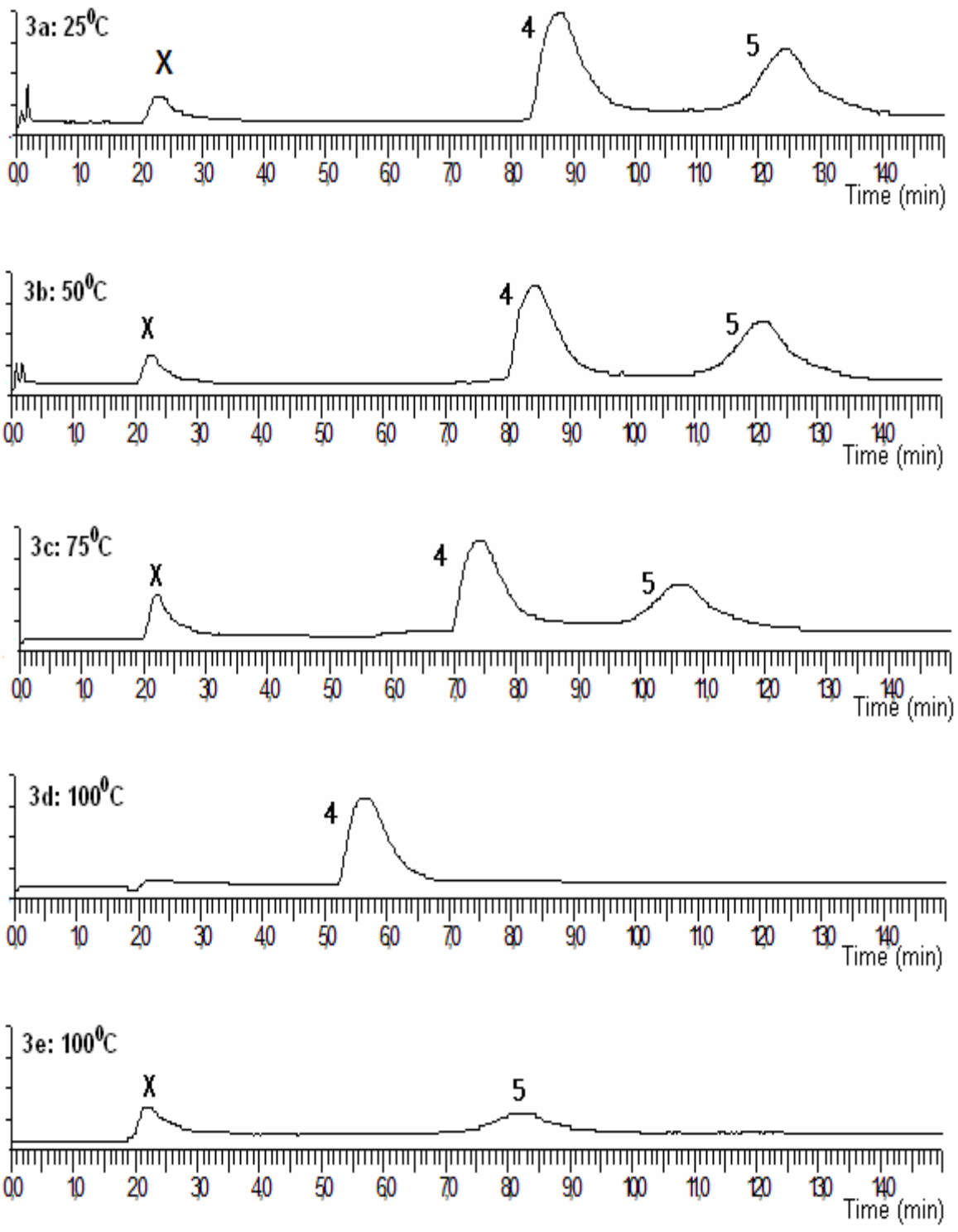


Figure 3a-e: Chromatograms of Mix II; albumin bovine (4) and apoferritin (5). The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size at 25ºC, 50ºC, 75ºC, and 100ºC. Mobile phase flow was 10 µl/min, 500 nl was injected and UV detection after separation was achieved at 25ºC at 280 nm. A gradient running from 30%-60% B in 15 minutes, followed by 60% -100% B in 5 minutes, and 100% - 35% B in 1minute was used. Mobile phase A was water + 0.1% TFA and mobile phase B was Acn + 0.1% TFA.

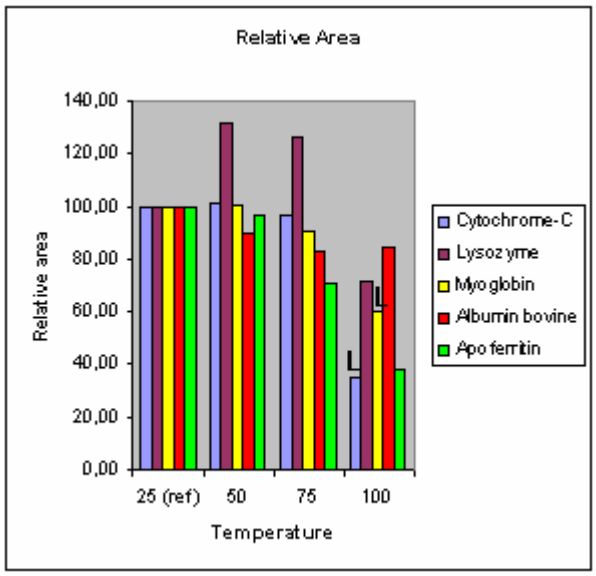


Figure 4: Relative areas of the five model proteins as function of temperature. L above a bar means that the area is significantly lower than the reference, according to a 95% confidence interval two tailed ANOVA test where n= 3.

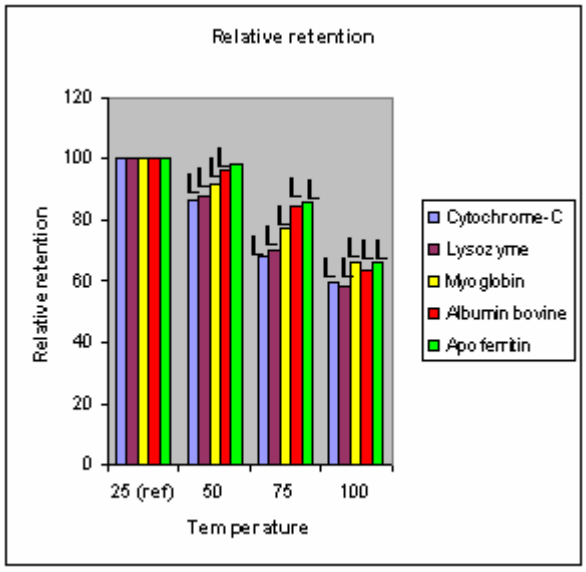


Figure 5: Relative retention of the five model proteins as function of temperature. L above a bar means that the retention time is significantly lower than the reference , according to a 95% confidence interval two tailed ANOVA test where n= 3.

The retention time decreased when the column temperature was increased as expected according to the Linear Solvent Strength model (LSS) [4]. The decrease was roughly the same for all the proteins tested, about 10-15% per 25 degree increase. The relationship between the retention factor and temperature should according to the theory be linear, that is, as long as the interaction surface between the analyte and the phases remains unchanged. In case of denaturation, the proteins unfold and expose different areas of different polarity to their environment, which means that the interactions between protein and stationary phase may change. In such a case, a linear relationship will no longer necessarily be expected. This can be visualized by a Van't Hoff plot [4, 22, 23], which shows ln k versus inverse temperature (fig 6). The proteins in Mix I; cytochrome-C, lysozyme, and myoglobin show a linear behaviour over the entire temperature range, while the proteins in Mix II; albumin bovine and apoferritin tend to diverge from linearity between 75ºC and 100ºC.

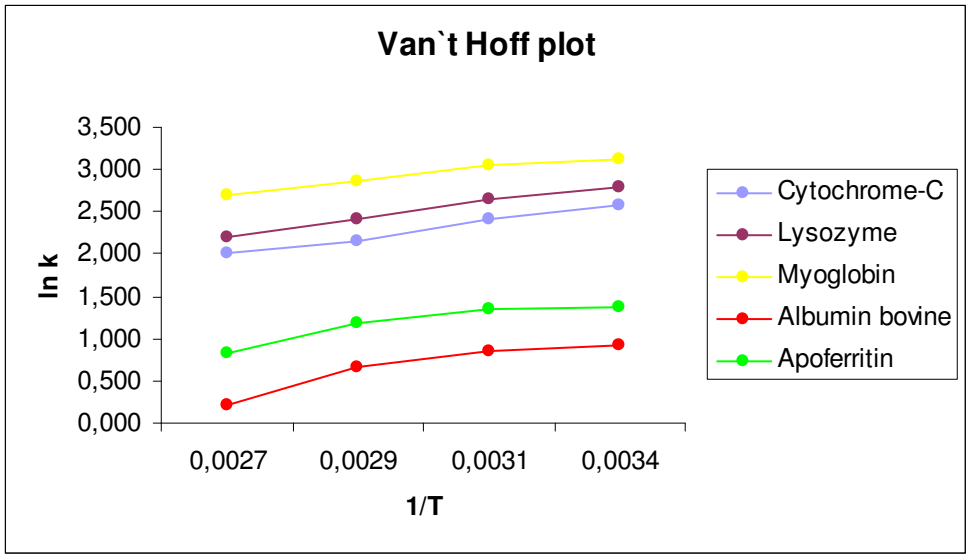


Figure 6: Van't Hoff plot for five proteins over the temperature range 25ºC-100ºC. Data obtained from the experiments are described in figure 2 and 3.

As mentioned, there is no exact temperature where the proteins suddenly denaturate, the denaturation process takes place gradually over a temperature interval, leaving a fraction of proteins in the native state. The temperature where 50% of the protein is denaturated is called the protein's melting point [8]. If the protein denaturate and the denaturation consequently results in a new conformation, a new peak should appear in the chromatogram. This peak should increase as the temperature arises due to an increase in the fraction of denaturated proteins. Meanwhile the peak representing the native state protein should decrease [13]. Such

peaks were not observed during this experiment (figure 2 and 3), indicating that if denaturation occurs, no new characteristic formation is shaped. The denaturation process provides random structures for the proteins investigated.

As for the recovery (figure 4), only myoglobin and cytochrome-C show a significant decrease in recovery at 100ºC relative to 25ºC. Large spread of the data and probably too few replicates prevent conclusions to be made at a 95% confidence level (See appendix A).

There are several possible explanations for the poor repeatability. First, the protein structure is very sensitive to changes in the environment. As mentioned in the introduction part, the native state is not a static conformation, but a dynamic structure with several internal movements between the different parts of the protein. Such changes may alter the state of interactions between the analyte and stationary phase.

Regarding denaturation, there are in general two factors that affect the process, the temperature and the change in environment between the mobile and the stationary phase. In theory, the temperature interval from no denaturation to 100% denaturation is small [10], but when rapid changes in the environment caused by interactions from both the polar mobile phase and the unpolar stationary phase happen simultaneously, this may not be the case. Furthermore, the denaturation process is not very well understood and there is a lack of general rules for the unfolding of different proteins. A mixture of denaturated, partly denaturated, and denaturated proteins may therefore yield different contributions to the molar absorbtivity. Another possible contribution to uncertainty in the peak area determination is the manual setting of the starting and ending points of the chromatogram peaks. Since the retention times changed during this experiment, it was difficult to determine the peaks automatically. This was therefore done manually, and the starting and ending points might have been set a slightly differently each time contributing to errors in the total peak area. An increase in the number of replicates may probably improve the certainty of the experiment, although this is a time consuming process.

4.2 **Denaturation outside the column:**

To determine the approximately melting point of the proteins and to study the proteins' possible ability to refold after being denaturated, the proteins were heated before being chromatographed. The proteins were heated to 50ºC, 75ºC, and 100ºC by applying 100 µl of protein mixture in a microcentrifuge plastic cup on a glass plate for 60 minutes in an oven equal to the one used to heat the column in these experiments. A non heated sample was used as reference. After being analyzed the first time, the rest of the protein mixture was cooled at 4ºC for 24 hours. The mixture was then chromatographed again to see if any denaturated protein had refolded to the original state. The peak areas obtained for the heated/cooled samples were determined and the relative areas and recovery are presented in figure 7a and b.

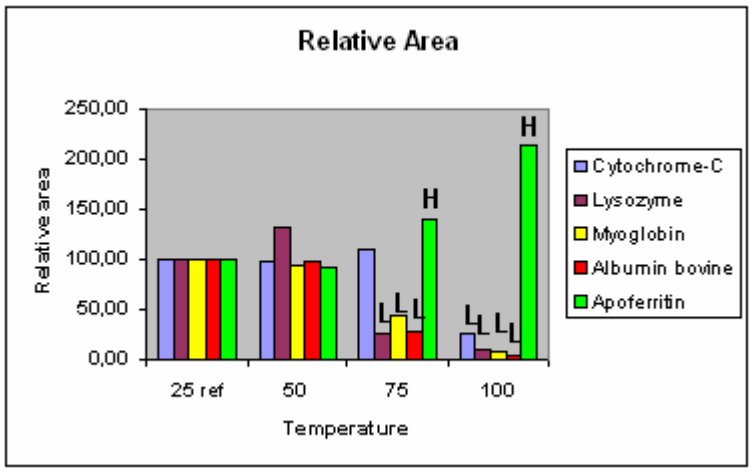


Figure 7a: Relative areas of 5 proteins after being heated for 1 hour. The proteins were heated to 500, 750, and 100ºC for 1 hour prior to injection. The area at 25ºC, in which proteins stored at room temperature for 60 minutes, is used as reference. The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 25ºC at 214 nm for Mix I and 280 nm for Mix II. Mix I was analysed using a linear gradient running from 30% -33% B in 5 min, then 33% -50% B in 6 min, and 50% -30% B for 1 min. Mix II: 35% -55% B in 10 min and 55% -35% B for 1 minute. Mobile phases were A; water + 0.1% TFA and B; Acn + 0.1% TFA. L above a bar means significantly lower than the reference. H above a bar means significantly higher than the reference, according to a 95% confidence interval two tailed ANOVA test where n= 3.

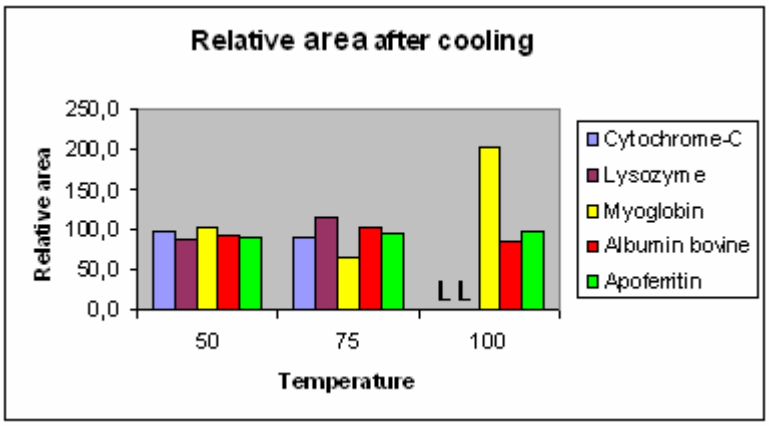


Figure 7b: Peak area after 24 h cooling relative to peak areas obtained from proteins being chromatographed right after heating. The same samples that were used in 7a had been stored for 24 hours after heating before being chromatographed once more under the same conditions as in 7a. L above a bar means significantly lower than the reference according to a 95% confidence interval two tailed ANOVA test where n= 3.

As seen from Figure 7a no significant differences are observed below 75ºC. At 100ºC some of the replicates did not show any peaks at all for some of the proteins, however these were still accounted for when the average result was calculated (area = 0). Generally very large variations between replicate areas were observed during this experiment, especially at higher temperatures (Appendix B). All proteins except apoferritin showed a decrease in peak area at higher temperatures. Apoferritin shows a significant increase in peak area indicating that something is happening when the temperature is increased. The possibility of an overlap from other denaturated proteins from the same mixtures was excluded by repeating the experiment with single proteins. A similar result was obtained. This unexpected behaviour can not be explained at present. It should be noted that apoferritin is the largest protein and has the lowest pI value of the investigated proteins (Table 1). Further experiments should be performed to confirm any possible tendencies of increased recovery for this protein, also above 100ºC.

The recovery for proteins chromatographed after having been cooled for 24 hours is presented in table 7b. If the proteins had refolded, the peak areas after cooling should be higher than the peak area directly after heating (relative area above 100). If no refolding had occurred, the areas would have been expected to be the same before and after heating (relative area =100). None of the proteins investigated show a significantly increase in area after being cooled. Some of the proteins even show a significant decrease in recovery after cooling, indicating a further destruction of the native protein structure. A failure of renaturation for most proteins

are not really surprising since protein (re)folding in biological systems is generally a highly enzyme aided process [8-9].

At higher temperatures in particular, the repeatability was bad (Appendix B), limiting the ability to draw conclusions.

4.3 **The consequence of increased residence time:**

Protein denaturation caused by high temperature is not only determined by the actual temperature, the time the protein is exposed to the temperature will also play a part [8-9]. Proteins that are heavily retained on a hot column are exposed to the high temperature for a longer time than proteins that are eluted fast. The time the proteins are exposed to the actual oven temperature while dwelling inside the oven is referred to as residence time. As discussed in the introduction part, proteins are generally difficult to separate and separation of complex mixtures may take a long time, resulting in long residence time [3-7]. Separation at different residence times at different temperatures were performed to see how the residence time affects the recovery. The model proteins were "trapped" inside the oven using a low strength mobile phase containing 10% B for 5, 10, 30, and 60 minutes (delay period) at 25ºC, 50ºC, 75ºC, and 100ºC before starting a relatively fast eluting gradient. Since the proteins respond differently to the mobile phase composition at different temperatures, different mobile phase gradients were used to provide roughly the same retention times after the delay period. The actual gradients are described in figure text 8.

Proteins chromatographed with no delay were used as reference. Figure 8 shows the relative areas obtained at different temperatures (a-d) as function of delay period.

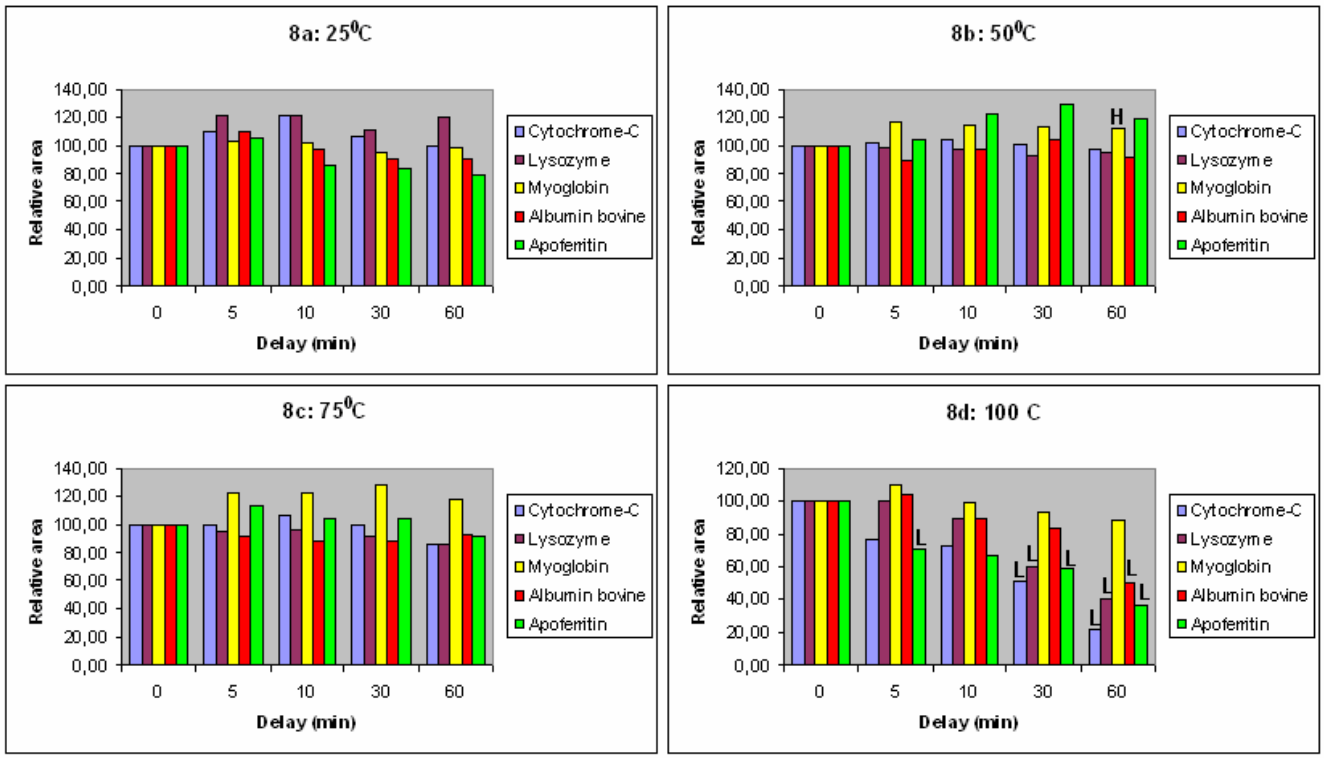


Figure 8a-d: The effect of residence time at 25ºC, 50ºC, 75ºC, and 100ºC for model proteins. The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 25ºC at 214 nm for Mix I and 280 nm for Mix II. The proteins were delayed for 5, 10, 30, and 60 minutes by using mobile phase consisting of 10% B before starting the actual gradient. Gradients were: ***25ºC Mix I****; 30% -33% B in 5 minutes, 33% -50% B in 6 min, and 50% -30% B in 1 minute****. 25ºC Mix II****; 35% -55% B in 10 min and 55% -35% B in 1 min.* ***50ºC Mix I;*** *30% -35% B in 10 min, 35% -50% B in 2 min, and 50% -30% B in 1 min.* ***50ºC Mix II;*** *30% -50% B in 10 min and 50% -30% B in 1 min.* ***75ºC Mix I;*** *28% -31% B in 7 min, 31% -50% B in 2 min, and 50% -28% B in 1 min.* ***75ºC Mix II;*** *30% -50% B in 10 min and 50% -30% B in 1 min.* ***100ºC Mix I;*** *24% -26% B in 10 min, 26% -50% B in 5 min, and 50% -24% B in 1 min.* ***100ºC Mix II;*** *30% -50% B in 10 min and 50% -30% B in 1 min. Mobile phase A was water + 0.1% TFA. Mobile phase B was Acn + 0.1% TFA. L above a bar means significantly lower than the reference. H above a bar means significantly higher than the reference, according to a 95% confidence interval two tailed ANOVA test where n= 3.*

For the proteins investigated, a delay period of 60 minutes or less did not have any significant effect on the relative peak areas at temperatures below 100ºC. At 100ºC, residence times exceeding 30 minutes tend to cause a significant decrease in the peak area for most proteins investigated. An exception is myoglobin, which seems to withstand long time temperature exposures quite well. Note that the results obtained show potential differences relative to a short time exposure and not a no time exposure since the reference proteins also have to pass through the column to be separated. Some degree of denaturation may therefore occur even for the reference proteins. Typical retention times without delay were 4-10 min (see appendix C).

4.4 Comparisons of mobile phase gradient and temperature gradient:

Alternatively to maintaining a constant temperature, the oven has the ability to increase the column temperature during the separation period, providing a temperature gradient. A temperature gradient and a mobile phase gradient were developed and compared as for the ability to separate the model proteins.

Two temperature gradients that provided good separation for Mix I and Mix II respectively was developed and compared with relatively fast mobile phase gradients with respect to protein peak areas. Table 3 shows the results. Figures 9 and 10 show the different chromatograms obtained for Mix I and Mix II respectively.

Table 3: Comparison of peak areas of proteins using mobile phase gradient and temperature gradient elution. The mobile phases were A; water + 0.1% TFA and B; Acn + 0.1% TFA. Peak areas represent the recovery of the proteins. N means no significant differences at 95% confidence level using tow-tailed ANOVA. See figure 9 and 10 for chromatograms.

Protein	Area Mobile phase gradient	Area temperature gradient	Difference %
Cytochrome-C	77075	70844	1,63 N
Myoglobin	96167	111178	15,61 N
Lysozyme	84436	77850	-7,80N
Albumine bovine	136351	144726	6,14 N
Apoferritin	126988	138050	8,71N

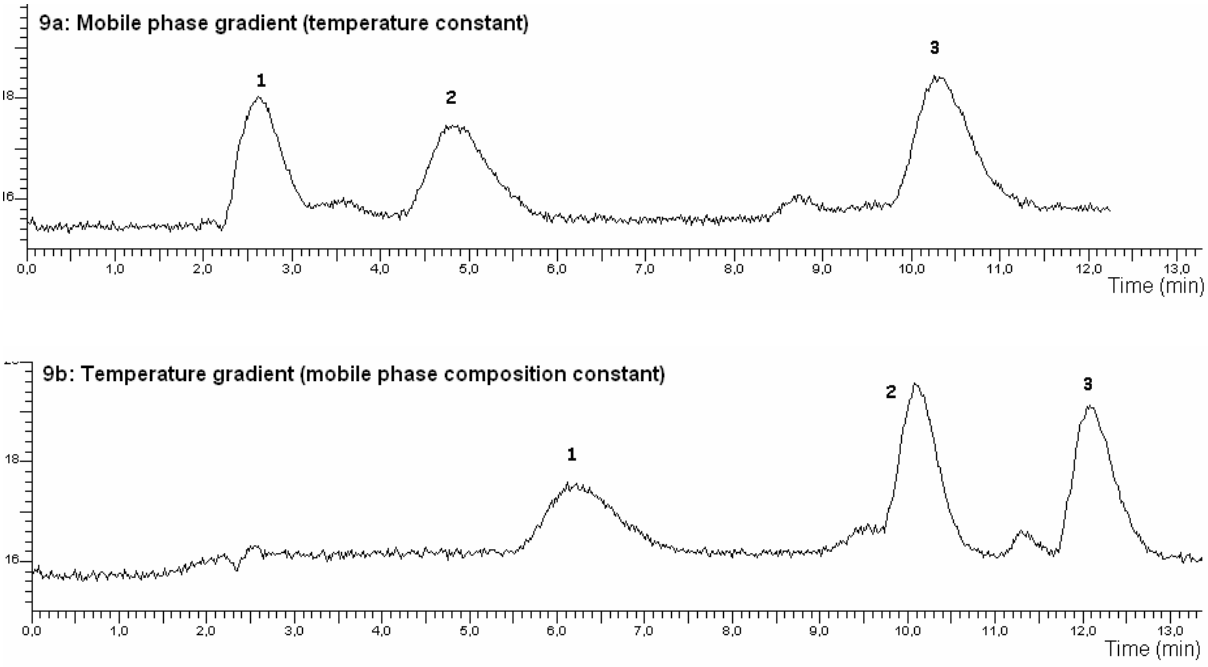


Figure 9a-b: Chromatograms of Mix I; cytochrome-C (1), lysozyme (2), and myoglobin (3) using mobile phase (a) and temperature (b) gradient elution. The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 25ºC at 214 nm. In chromatogram 9a, the temperature was isotherm 25ºC, mobile phase gradient was 30% -33% B in 5 min, 33% -50% B in 6 min, and 50% -30% B in 1 min. In chromatogram 9b the mobile phase composition was isocratic 33% B, temperature gradient was 25ºC-40ºC in 6 min, 40ºC-100ºC in 2 min, and remaining at 100ºC.for 1 min. Mobile phase A was water + 0.1% TFA. Mobile phase B was Acn + 0.1% TFA.

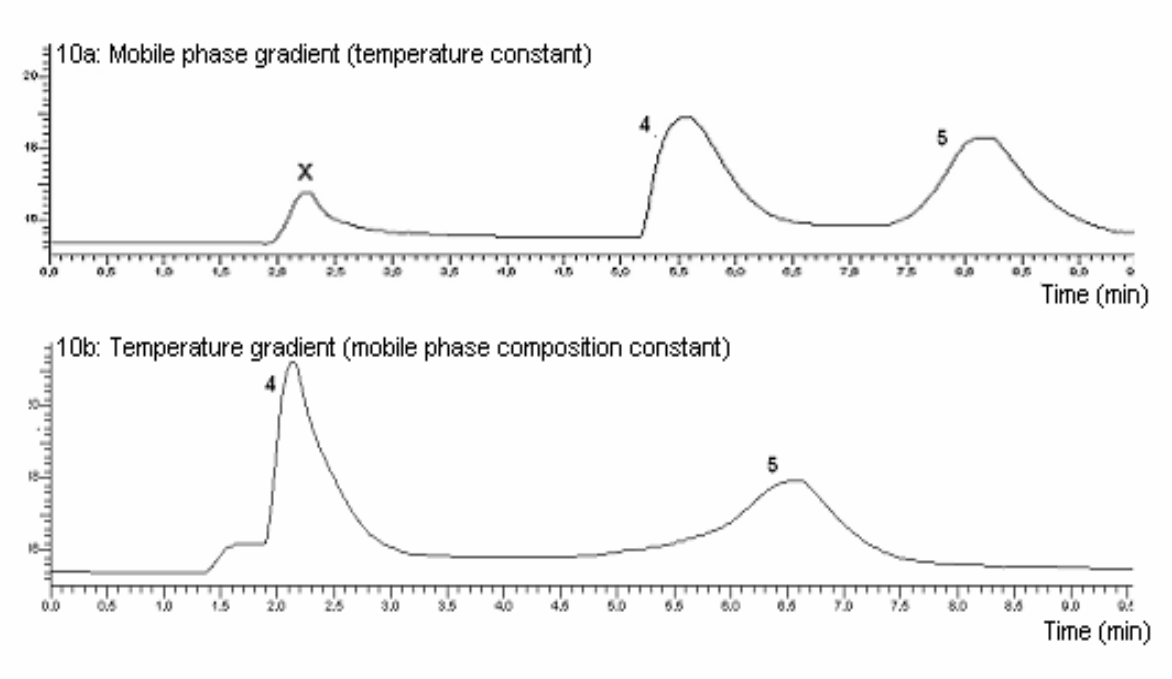


Figure 10a-b: Chromatograms of Mix II; albumin bovine (4) and apoferritin (5) using mobile phase (a) and temperature (b) gradient elution. The separation was carried out on a PLRP-S column, 150 x 0.3 mm, 5 µm particles, and 1000 Å pore size. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 25ºC at 280 nm. In 10 a, the temperature was isotherm 25ºC, mobile phase gradient was 35% -50% B in 5 min and 50% -35% B in 1 min. In 10b the mobile phase was isocratic at 40% B. The temperature gradient was 30ºC-100ºC in 5 min and constant at 100ºC for 1 min. Mobile phase A was water + 0.1% TFA. Mobile phase B was Acn + 0.1% TFA.

It is shown in figure 9 and 10 that both a temperature gradient and a mobile phase gradient are able to complete the separation of the proteins in Mix I and Mix II. Visually, the chromatograms show that the relative retention between the proteins when using the temperature gradient is different than when using the mobile phase gradient. This indicates that changes in mobile phase compositions and changes in temperature affect the different proteins in different ways. By further optimization both gradient modes would probably provide even faster separations. However, optimizing the gradients was not the aim of this study. The purpose was only to show that it is possible to separate the proteins using both kinds of gradients. The peak areas obtained showed no significant differences for any protein investigated.

4.5 **Temperature effect on separation of a complex mixture:**

In the following experiments involving a complex protein mixture (4.5 -4.7), a monolithical column has been selected. Monolithical columns are a relatively new types of columns which have proven to be promising for reversed phase separation of proteins [24]. While traditional LC columns are packed with microsize particles resulting in a relatively large void volume between the particles, the packing material in monolithical columns consists of one piece of rigid porous polymer with no interstitial volume [25-26]. Due to a lack of intraparticular volume the contact surface between the mobile and stationary phases is increased resulting in a more efficient mass transport between the phases and thereby high efficiency even at high speed [27-28]. A master thesis on production and testing of polystyrene based monolithical columns has been performed at the University of Oslo, Department of Chemistry (2004-2006) [18], and it was of interest to test one of these columns' ability to separate proteins at higher temperatures as well.

The ability to improve or possibly fulfil an almost complete separation of a complex protein mixture by optimizing the column temperature was investigated.

A mobile phase gradient that almost separated the proteins was developed, and the separation was carried out with temperatures from 30ºC-100ºC (isotherm) and with a temperature gradient running analogous with the mobile phase gradient. Figure 11a-e shows the chromatograms for Mix III being chromatographed under different temperature conditions using the same mobile phase gradient.

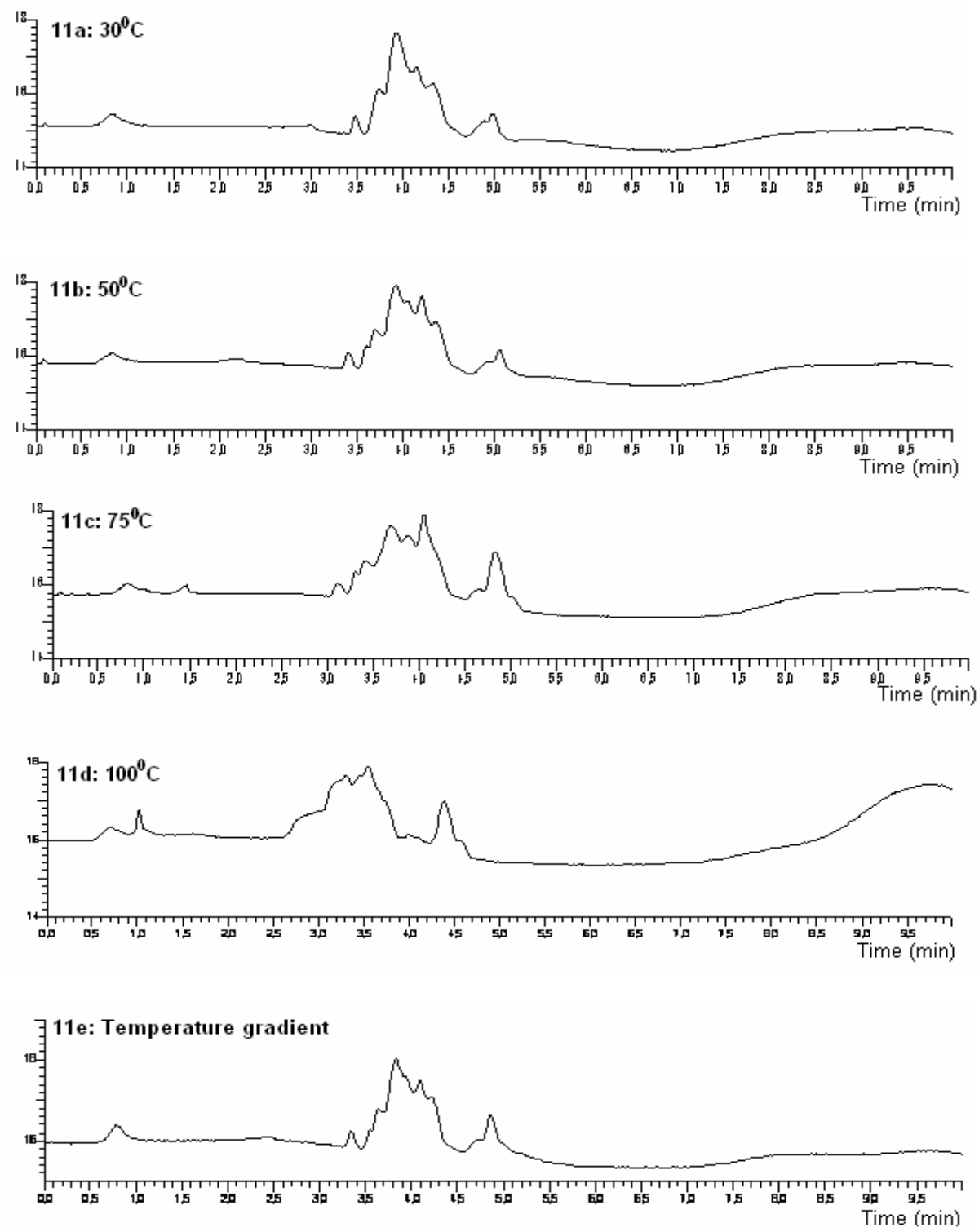


Figure 11 a-e: Separation of protein Mix III as function of temperature. Separation was carried out on a divinyl-benzene monolithic column 60 x 0.180 mm. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection was achieved at 214 nm. 3 replicates ands a blank run were performed at each temperature. A mobile phase gradient running from 20%-70% B in 5 min, 70% -100% B in 1 minute, and 100% B for 2 minutes was used. Mobile phase A was water /Acn /TFA (98 /2/0.1% V/V) and mobile phase B was water/Acn/TFA (5 /95 /0.1 v/v). In 11e, a temperature gradient running from 30ºC-100ºC for 10 minutes was carried out simultaneously with the mobile phase gradient.

Baseline separation was not achieved at any temperature or by performing the temperature gradient for Mix III.

When trying to develop a slower mobile phase gradient in order to successfully complete the separation, the peaks appeared too small relatively to baseline noise. The concentration of the proteins in Mix III was in the range 0.05-0.5 mg/ml, this is probably too low to gain acceptable peaks using a slower gradient for the given instrumentation and conditions.

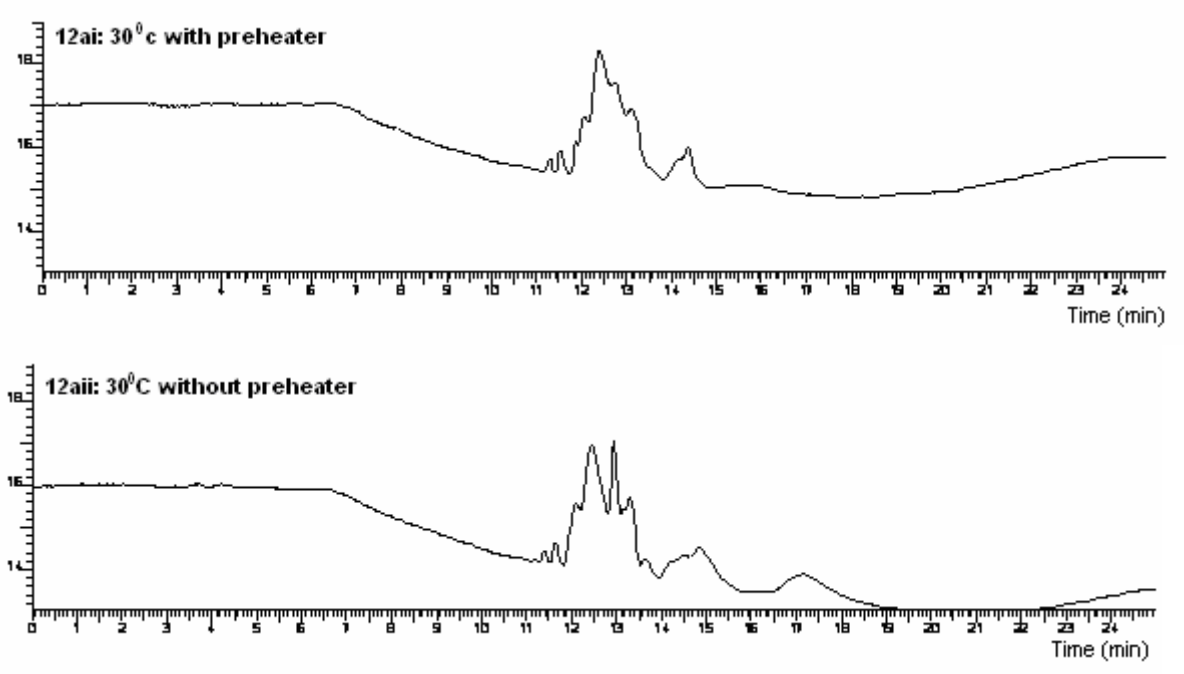
Due to overlaps, misshaping of peaks, tailing, and the fact that the retention time, peak intensity, and peak shape for the individual protein may have changed as function of temperature, it was very difficult to see the number of actual peaks in the different chromatograms. Furthermore, the proteins have not been investigated for possible peaks caused by stabile denaturated formations under the actual conditions [13]. Some of the observed peaks may be of this kind. This could for example explain the increasing intensity of the peak(s) appearing around 4.5-5 minutes as temperature increases, since such structures should be expected to have a longer retention time relative to their corresponding native state protein due to a more unpolar surface and thereby stronger interactions with the stationary phase (se introduction). Since we are dealing with a mixture of known proteins, injection of single proteins could reveal the appearance of possible peaks caused by denaturated proteins. This is a very time consuming operation even for a mixture containing only ten known proteins. Such injections were not performed.

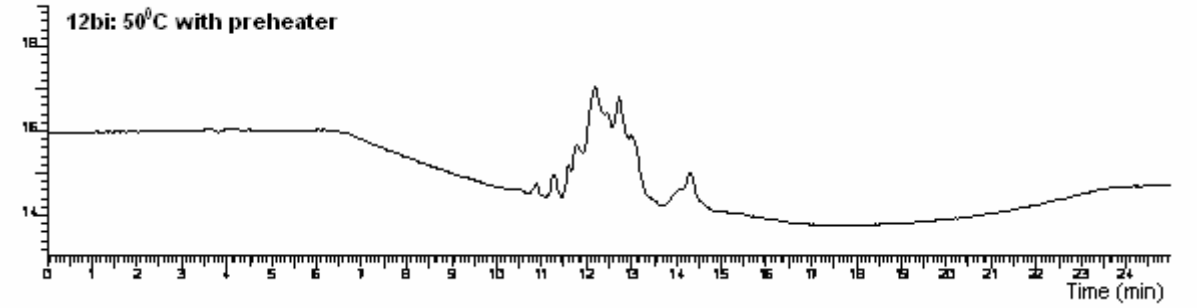
Since different proteins respond differently to temperature changes, the separation achieved may not necessarily be improved at higher temperatures. Two proteins separated at one temperature may overlap at another temperature, higher or lower. Considering this is a mixture containing 10 proteins, there is room for many possible overlaps.

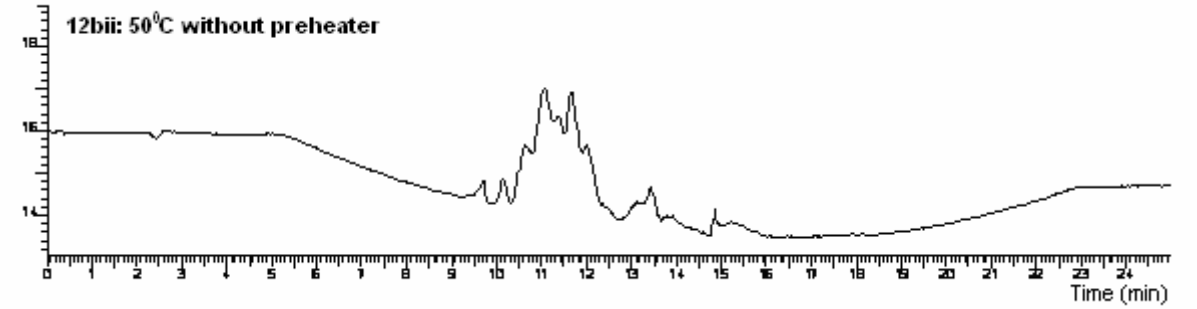
4.6 **The preheater effect:**

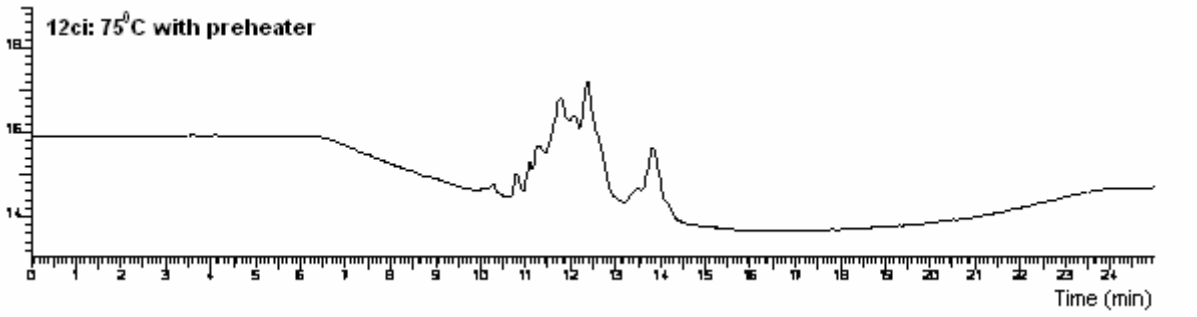
By applying a preheater prior to the system (see instrumental), the mobile phase is heated to the same temperature as the column temperature before reaching the column, preserving a constant mobile phase temperature through the column, creating more stabile and probably more repeatable conditions for the separation. On the other hand, the less time the proteins are exposed to high temperature, the less denaturation is expected [8-9]. By disconnecting the preheater the proteins are exposed to high temperature for a shorter period of time, at least for a few seconds. On the other hand, the mobile phase containing the proteins may not reach the actual oven temperature at all before leaving the heated zone if the preheater is not applied. This will complicate the retention process, at least in a theoretical approach. By performing a separation under the same conditions, only replacing the preheater with a capillary and compare the results as for separation ability, peak areas, and repeatability, the effect of using a preheater is examined.

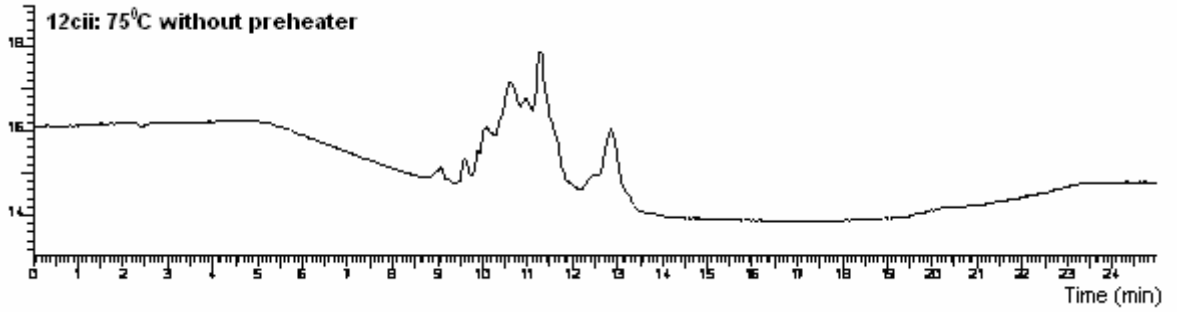
Mix III was chromatographed at different temperatures, using a temperature gradient with and without the preheater applied. The chromatograms are shown figure 12a-f.

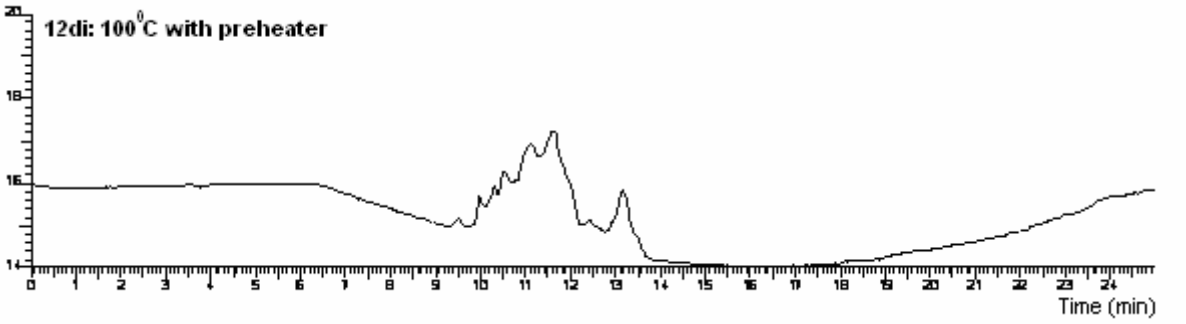


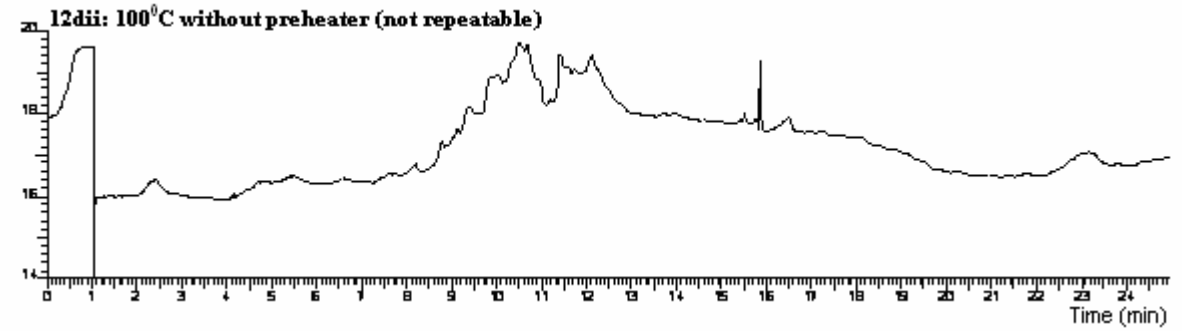

12bi: 50^{0}C with preheater
Time (min)

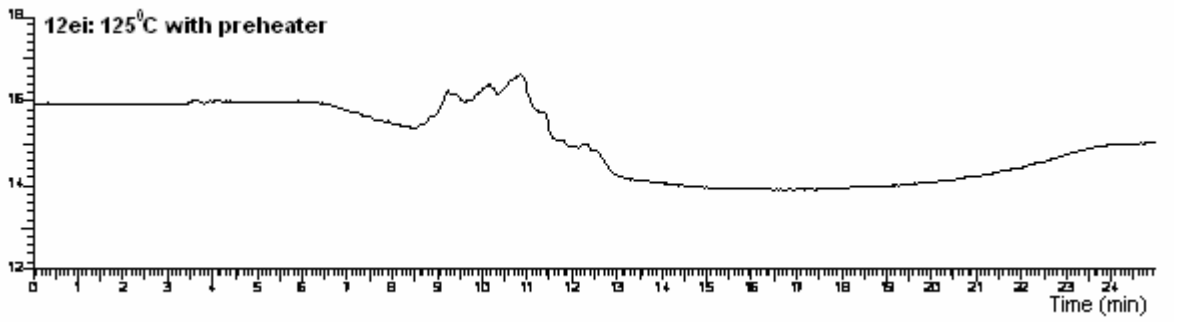

12bii: 50^{0}C without preheater
Time (min)

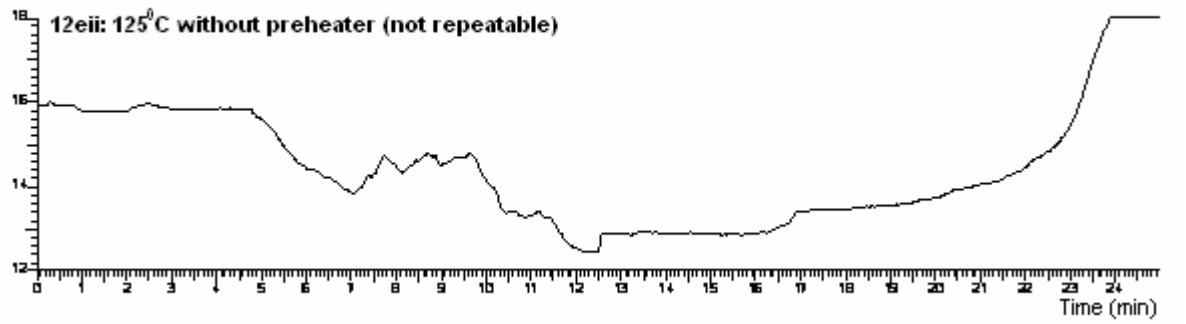

12ci: 75^{0}C with preheater
Time (min)


12cii: 75^{0}C without preheater
Time (min)


12di: 100°C with preheater
Time (min)


12dii: 100°C without preheater (not repeatable)
Time (min)


12ei: 125°C with preheater
Time (min)


12eii: 125°C without preheater (not repeatable)
Time (min)

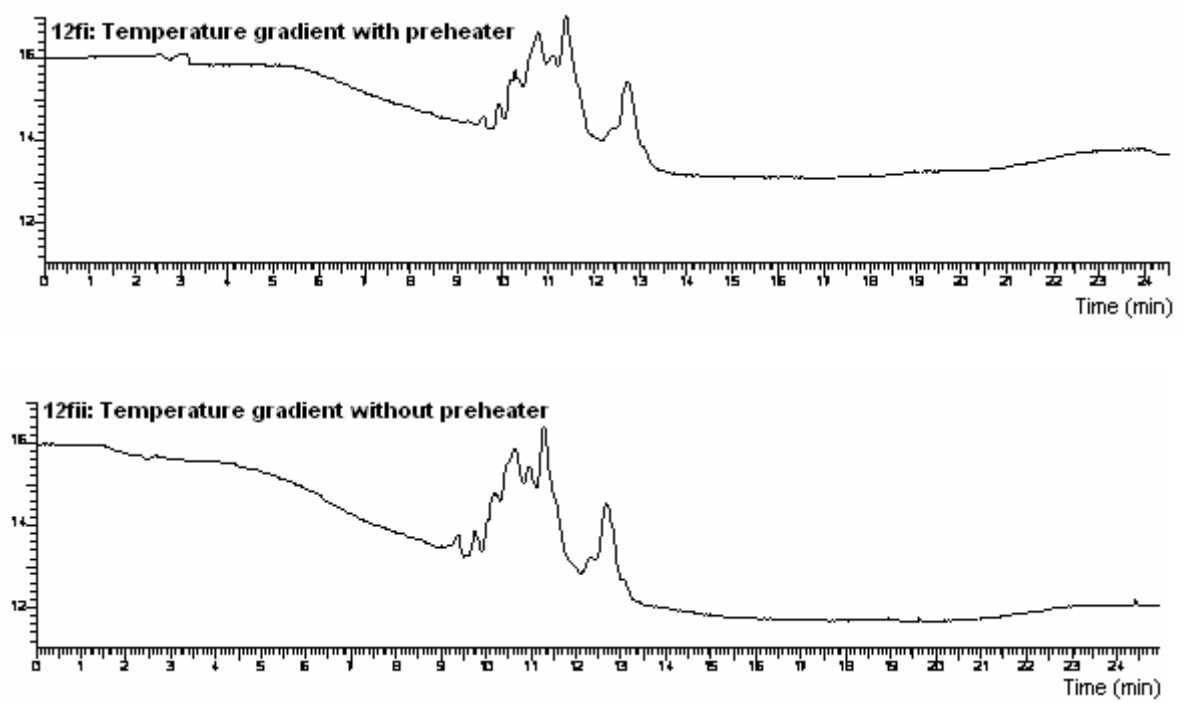


Figure 12a-f: Comparison of separation at different temperatures with and without the preheater added for separation of Mix III. The separation was carried out on a divinyl-benzene monolithic column 60 x 0.180 mm. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 214 nm. 3 replicates and a blank run were performed at each temperature. Mobile phase A was Water /Acn /TFA (98 /2/0.1% V/V) and mobile phase B was water/Acn/TFA (5 /95 /0.1 v/v). A gradient going from 0% -100% B in 20 minutes and remaining at 100% for 5 minutes was used. . In 12f, a temperature gradient running from 30ºC-100ºC for 20 minutes was carried out in parallel with the mobile phase gradient

The retention times are not comparable since the preheater and the replacing capillary do not share the same dimensions (see experimental).

At lower temperatures, it seems that the best separation is achieved without the preheater. At such temperatures, the temperature difference between the mobile phase, which is stored in reservoirs at room temperature (approximately 20ºC), and the oven is small and the mobile phase temperature increase may be considered insignificant. When the temperature difference was large due to higher oven temperatures, the observed differences were smaller. The observed improvement in separation when no preheater was applied tended to be especially significant at 30ºC but can also be observed at 50ºC and 75ºC. The effect of applying a preheater versus not applying a preheater was expected to be most notable at higher

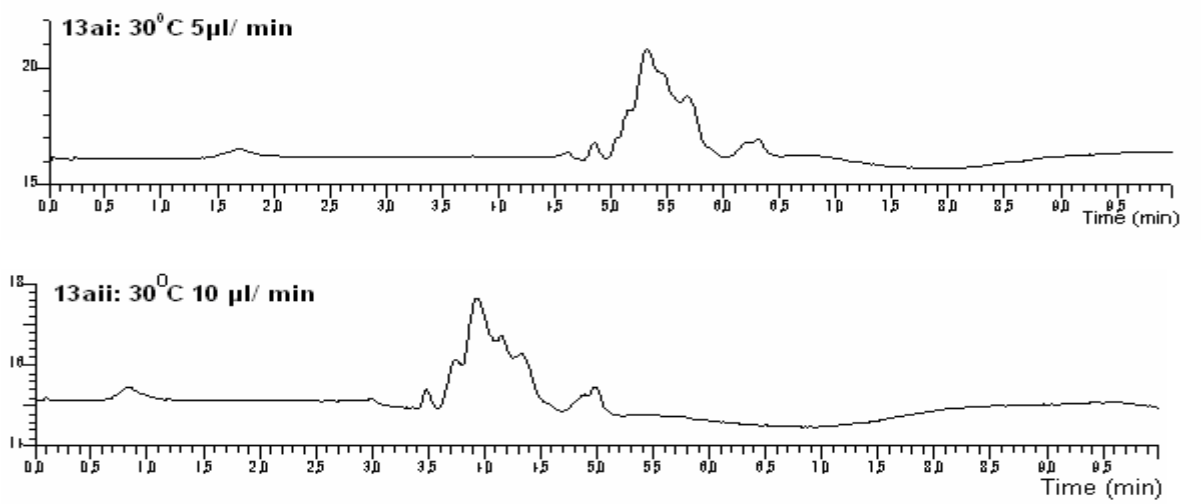
temperatures and not at lower temperatures, as observed. No explanation for this surprising result is given.

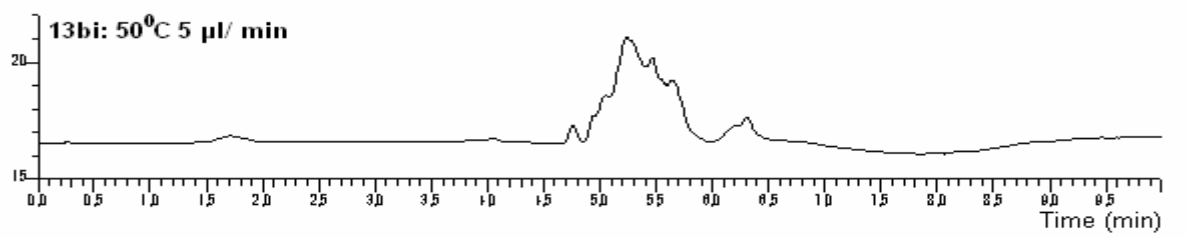
At higher temperatures, 100ºC and 125ºC, the chromatograms between replicates were no longer repeatable without the preheater attached. In order to achieve repeatable data for this mixture at high temperatures, the preheater had to be applied.

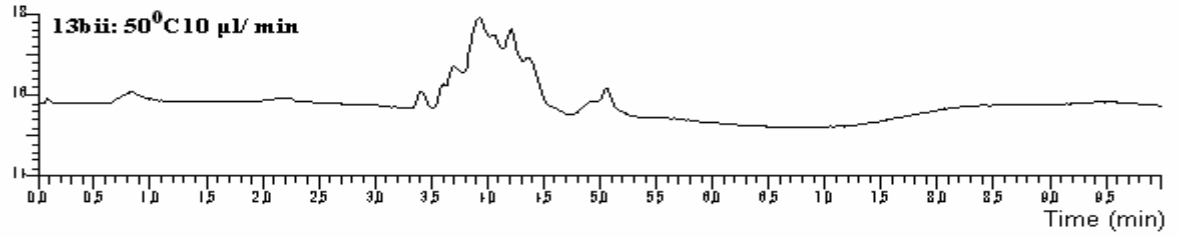
The chromatograms achieved using the temperature gradient did not show any notable differences.

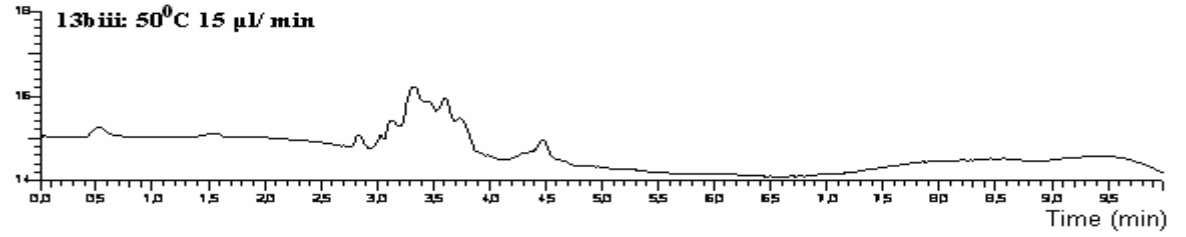
4.7 The effect of mobile phase flow rate:

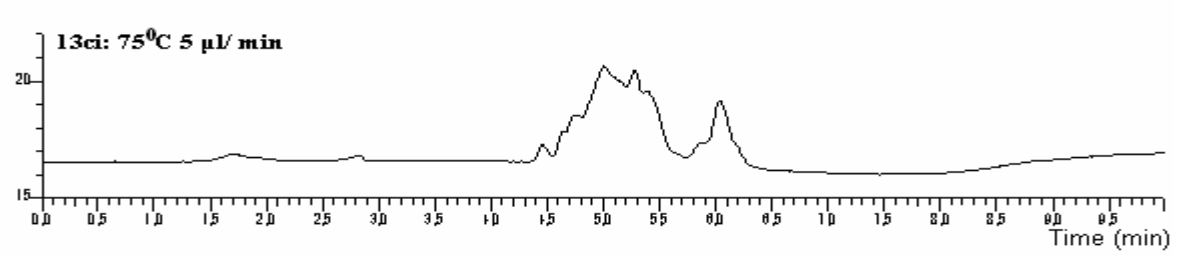
Monolithical columns are known for being able to gain high efficiency even at higher flow rates [26]. It was investigated if the separation could be improved by increasing the flow rate. Mix III was separated under the same conditions, only changing the mobile phase flow rate and temperature. The chromatograms are shown in figure 13a-d.

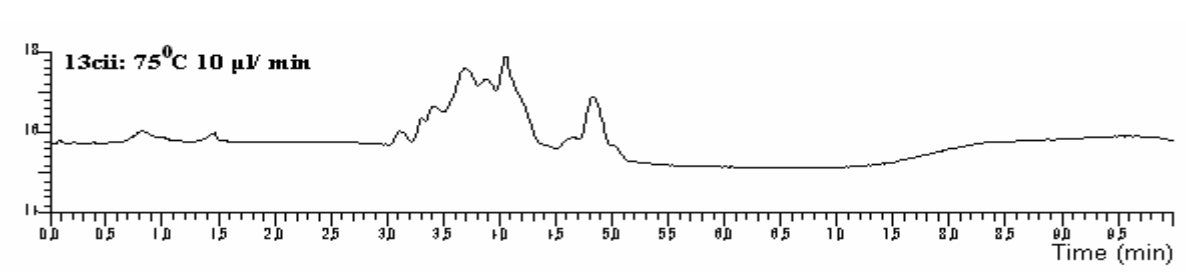


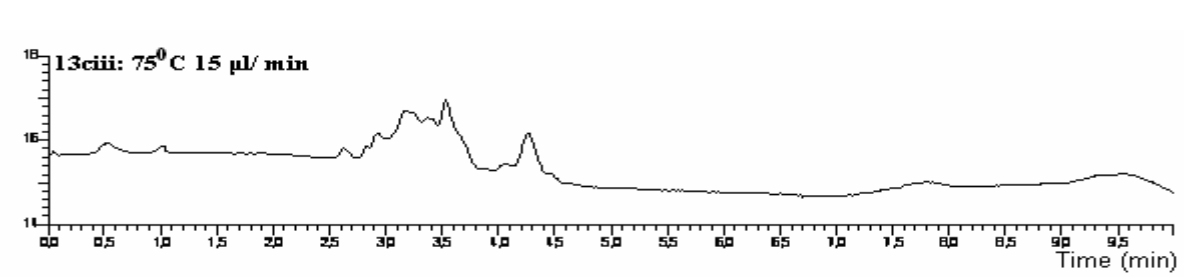

13bi: 50^{0}C 5 µl/ min
Time (min)


13bii: 50^{0}C10 µl/ min
Time (min)


13biii: 50^{0}C 15 µl/ min
Time (min)


13ci: 75^{0}C 5 µl/ min
Time (min)


13cii: 75^{0}C 10 µl/ min
Time (min)


13ciii: 75^{0}C 15 µl/ min
Time (min)

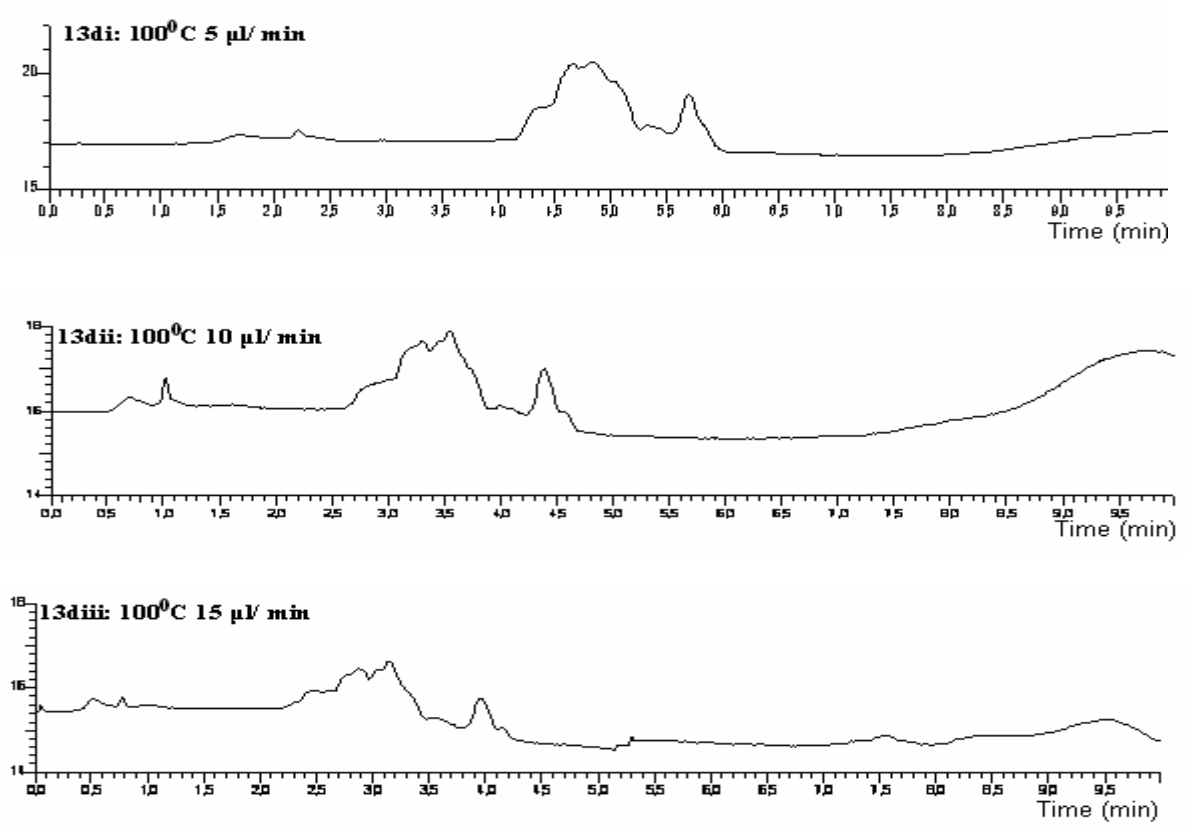


Figure 13 a-d: Comparison of different flow rates by separating Mix III at 30ºC, 50ºC, 75ºC, and 100ºC and mobile phase flow 5 µl/ml, 10 µl/min, and 15 µl/min. The separation was carried out on a divinyl-benzene monolithic column 60 x 0.180 mm. Mobile phase flow was 10 µl/min, 500 nl was injected, and UV detection after separation was achieved at 214 nm. 3 replicates and a blank run were performed at each temperature. A gradient running from 0-100% B in 20 minutes and remaining at 100% for 5 minutes was used .Mobile phase A was water /Acn /TFA (98 /2/0,1% V/V) and mobile phase B was water/Acn/TFA (5 /95 /0.1 v/v).

One of the advantages of applying high temperatures is the reduced system pressure. At 30ºC, the pressure exceeded the pump's upper pressure limit (400 bar) with a mobile phase flow of 15 µl/min.

At all temperatures investigated, the retention time decreases at higher flow, as expected. By examining the chromatograms visually, the separation tends to improve when the flow is increased from 5µl/min to 10 µl/min, although full separation is not achieved at any temperature or flow.

5. Conclusions:

The effect of column temperature of five individual proteins on recovery and retention time has been investigated. It was shown that the retention time for all the investigated proteins decreases when column temperature is increased. The recovery represented by relative peak areas did not show significant decrease for any of the investigated proteins below 100ºC. When the proteins were heated for one hour prior to injection, a significant decrease in relative area was observed already at 75ºC. A possible explanation for this decrease at lower temperature than in the first experiment may be that the proteins in the first experiment were exposed to the actual temperature for a period shorter than 60 minutes. When being heated in advance, apoferritin showed a significant increase in recovery from 75ºC and up. No behaviour similar to this was observed for apoferritin in any other experiment conducted in this thesis.

Regarding residence time in the system, the data indicates that exposure to higher temperatures does not cause further denaturation for at least 60 minutes at 75ºC or below for the proteins investigated. At 100ºC, a delay beyond 30 minutes causes significantly lower recovery for most proteins investigated. The exception is myoglobin that did not show any significant decrease at all.

The overall results indicate that the proteins investigated denaturate around 75ºC-100ºC and that exposure time at 100ºC is an important parameter, while it does not seem to matter at temperatures below 75ºC for at least 60 minutes. Measuring peak areas at different temperatures with 25 degrees difference is most likely not a good way to observe protein

denaturation. It is likely that only four measurements with 25 degrees difference are too few to investigate a temperature span from 25ºC-100ºC. At least in the high temperature range of 75ºC-100ºC, more tests should be performed investigating more temperatures in this range. Monitoring the response from a UV detector does not provide any structural information as for investigation of folded versus denaturated proteins. According to theory, an unfolded protein should maintain a totally different surface as for polarity and should therefore interact differently with the stationary phase [4, 8-9] A Van't Hoff plot can be drawn to recognize such changes [22-23]. It was shown that two out of five proteins had a point at 100ºC that diverged from linearity.

It was shown that Mix I and Mix II could be separated using a temperature gradient. By comparing the peak areas with a separation performed using a mobile phase gradient, no significant differences in the peak areas was found. Temperature gradients are rarely used by themselves, but are usually accompanied by a mobile phase gradient. The combination of using both mobile phase and temperature gradients simultaneously was tested using a monolithical column to separate a complex protein mixture. The separation was accompanied and compared with several other attempts to separate the same mixture using different temperatures and mobile phase flow rates. None of the attempts to complete the separation of all the ten proteins were successful, but differences were observed. The protein concentration of the mixture (0.05-0.5 mg/ml) was probably to low for the equipment used. By repeating the experiments using higher concentrations, slower gradients, and higher flow, a complete separation could probably be fulfilled.

6. References:

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Part II:

Fractionation and separation of basic plasma proteins using two-dimensional liquid chromatography.

1. **Introduction:**

One of the earliest branches of biochemistry is the study of proteins. It has been known for over a century that the study of these macromolecules is a key to understand processes in living organisms on a molecular level [1].

Biological processes are often very complex and involve a large number of different proteins. For example, RNA polymerase II transcription complexes in eukaryotic ribosome consist of about 50 different proteins [2]. It is therefore necessary to identify a large number of proteins simultaneously to understand biological molecular processes. The protein composition may also change over time due to physical and chemical changes in its environment. The whole set of proteins that are present in a cell, tissue, or organism at one time is referred to as the proteome. The techniques that allow the study of the protein contents are know as proteomics [3].

The approach to efficient separation of proteins and peptides from complex mixtures began in the 1970's and has improved over the years, with two-dimensional gel electrophoresis (2D-GE) as the most common technique. Most separations of complex mixtures are today carried out using polyacrylamide gel electrophoresis (2D-PAGE) [4, 5], where the proteins are separated in two dimensions on a gel, according to charge via isoelectric focusing (IEF) and molecular size via sodium dodecyl sulphate (SDS) -PAGE. During the 1990's, mass spectrometry evolved to become the most important method for identification of proteins. New techniques as matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry [6-7], electrospray ionisation (ESI), and tandem mass spectrometry (MS/MS) [8-9] were introduced, and allowed atmospheric pressure ionisation and identification of macromolecules by MS. Mass spectrometry or tandem mass spectrometry is today the method of choice for identification of proteins after separation [10-11].

Although being the most common technique for proteomic separation, 2D-PAGE suffers from a number of limitations [3-5, 12-13]:

Firstly, parts of the proteome involving proteins that are very large, very small, highly hydrophobic, and/or have extreme pI values (<4 or >9), are poorly resolved in the gel.

Secondly, the dynamic range of most protein labels/stains is small compared to the dynamic range of a typical full proteome, limiting the ability to detect all proteins at the same time. Complex samples frequently require multiple gels to separate the entire range.

Finally, 2D-PAGE can not be directly coupled with MS. It requires manual (or robotic) intervention in order to remove the proteins from the gel and implement them for MS analyses.

An alternative to protein separation by gel electrophoresis is multidimensional liquid chromatography (LC^n) [14-18]. Many of the limitations of gel electrophoresis are not present in the multidimensional LC-methods, and liquid chromatography is therefore in many cases a better approach to proteomics than SDS-PAGE [3-4].

Many combinations of liquid chromatographic modes have been tested for this purpose; Size exclusion-reversed phase (SEC-RPLC) [15, 19-20], reversed phase-size exclusion (RPLC-SEC) [21], ion exchange-size-exclusion (IEC-SEC) [22], normal phase-reversed phase (NPC-RPLC) [23], and finally ion exchange-reversed phase (IEC-RPLC) [17-18, 24-25]. The latter is the most used combination [10, 13, 26].

In a two-dimensional system, transferring a fraction from the first to the second (analytical) column can be performed on-line or off-line, both methods carrying advances and limitations [4, 13, 27-28].

2. **Aim of study:**

Our research group at the University of Oslo, Department of Chemistry, has developed a capillary two-dimensional liquid chromatograph system that separates proteins from plasma according to pI and further by hydrophobicity [29-30].

In this study, an attempt to scale up this method in order to isolate proteins present at low concentration in a sample is presented. A system has been developed and separation of basic proteins from a plasma sample was selected to test the system.

3. **Experimental:**

3.1 **Measuring pH in the first dimension:**

The measuring of the mobile phase pH during a gradient run was performed both on-line and off-line. In both modes the same gradient running from 100% buffer A (pH ~ 11.0) to 100% buffer B (pH ~ 3.5) in 30 minutes and then remained at 100% B for 30 minutes, was used with a total flow of 1 ml/min. (See 3.2 for preparation of mobile phase A and B.) The pH of the eluent was measured as it eluted from the SAX column. In the on-line mode the pH gradient was monitored using a low volume pH flow cell equipped with a round tip double junction flow-through pH electrode from Amersham Biosciences AB (Uppsala, Sweden). The signal from the pH electrode was translated with a Cl-6507 pH amplifier and monitored with Science Workshop Data Studio software, both from PASCO (Rosewille, CA, USA). This software allowed simultaneous monitoring of the signal from the pH probe and the UV-detector.

In the off-line mode, 2 ml samples were collected for 60 minutes in vials and pH was measured using the same electrode. The results were presented as pH at 1, 3, 5 minutes

and so on, (Appendix E and F). The electrode was calibrated (3 point) prior to the on-line and off-line measurement. 3 replicates were performed in each mode.

3.2 Instrumentation and procedures:

Serum citrate plasma was achieved from Ullevål University Hospital (Oslo, Norway) and was diluted 1+5 with type I purified water. The water used for all mobile phases and preparation of samples and solutions was type I water purified using Milli-Q gradient system (non-TOC) water purification generating 18MΩcm ultrapure quality water from Milllipore S. A. S. (Molsheim, France).

The diluted plasma was injected by overfilling the external 1 ml loop of the injector from Valco instruments (Houston, TX, USA). The external loop was made of 1.0 mm i.d. x 31.85 cm (volume ≈ 1ml) PEEK tubing from Upchurch Scientific (Oak Harbor, Wa, USA).

Both pumps were gradient pump systems from Shimadzu (Columbia, MD, USA). They both consisted of two LC-10 AD pumps and a SCL 10A system control. All tubings used to connect the system were 1/16 in x 0.010 (0.25 mm) PEEK from Upchurch Scientific, and stainless steel ferrules were from Valco instruments.

The first dimension column was a PL-SAX (strong anion exchanger) polystyrene/divinylbenzene co-polymer with quaternary amine functionality, 8 μm particles, 1000 Å pore size, and dimensions 150x 4.6 mm from Polymer Laboratories (Shropshire, UK). The first dimension mobile phase was a salt buffer system consisting of piperazine 99% and dietanolamine >98.5%, both from Sigma Aldrich (St. Louis, MI, USA), and etanolnamine >99% from Fluka Chemicals (Buchs, Switzerland). A stock solution, 0.500 M of each salt was prepared in water. Appropriate dilution was done to obtain 0.100 M working solution, mobile phase A, pH ~ 11.0. Mobile phase B was similar, but was pH adjusted to pH~ 3.5 with diluted HCl 37% from KEBO lab (Oslo, Norway).

The separation was carried out by a gradient running from 0-100% B in 30 minutes, and then the system remained at 100% B for 30 minutes. The flow rate was constant 1 ml/min. An at least 30 min reequilibriation with 100% A was allowed between the runs. As the proteins were eluted from the column, they were transported to trap column 1. Both trap columns 1 and 2 were PLRP-S columns with 5 µm macroporous spherical polystyrene/divinylbenzene particles, 300 Å pore size, and dimensions 50 x 4.6 mm from Polymer Laboratories.

After complete trapping of the first fraction, the valve was switched (figure 1). The proteins eluted from the SAX after switching were trapped on trap column 2. Simultaneously, the flow through trap column 1 was now generated by pump 2 in the opposite direction. As the system was switched, fraction 1 was backflushed from the trap column and transferred to the analytical column for further separation. The analytical column was a PLRS-S column with 3 µm macroporous spherical polystyrene/divinylbenzene particles, 300 Å pore size, and dimensions 150 x 4.6 mm from Polymer laboratories. The second dimension mobile phase A consisted of water/Acn/TFA (95/5/0.1 v/v). Acetonitrile >99.8% was obtained from Merck (Haar, Germany) and TFA was UV spectroscopy quality >99% from Fluka Chemicals. Mobile phase B was Acn/water/TFA (95/5/0.1 v/v).

Second dimension separation was carried out by a gradient running from 0-80% B in 30 minutes. The flow rate was 0.5 ml/min if nothing else is specified. The proteins were detected using a SPD-10AV UV detector from Shimadzu operated at 280 nm. When the valve was switched back to the initial position, fraction 2 was likewise transferred to the analytical reversed phase column.

An alternative off-line procedure was also developed. The first dimension was carried out using the same equipment and gradients as described above. The eluted material from the first 10 minutes (basic fraction) was trapped on trap column 1, the rest was wasted. The procedure was repeated three times, trapping basic fractions from three injections. After

trapping the third fraction, the system was backflushed using Acn /water/TFA (85/15/0.1 v/v) at a flow of 1 ml/min. UV detection was used to monitor the elution. Approximately 2.5 ml of eluted material was collected in a polypropylene vial. The sample was reduced to about 50µl by using nitrogen gas with a purity of 2.6 (99.6%) from AGA (Oslo, Norway) for 90 minutes. All the material was injected using the same injection system as described above, but was transferred directly to the reversed phase column. The UV-detection was performed at 280 nm.

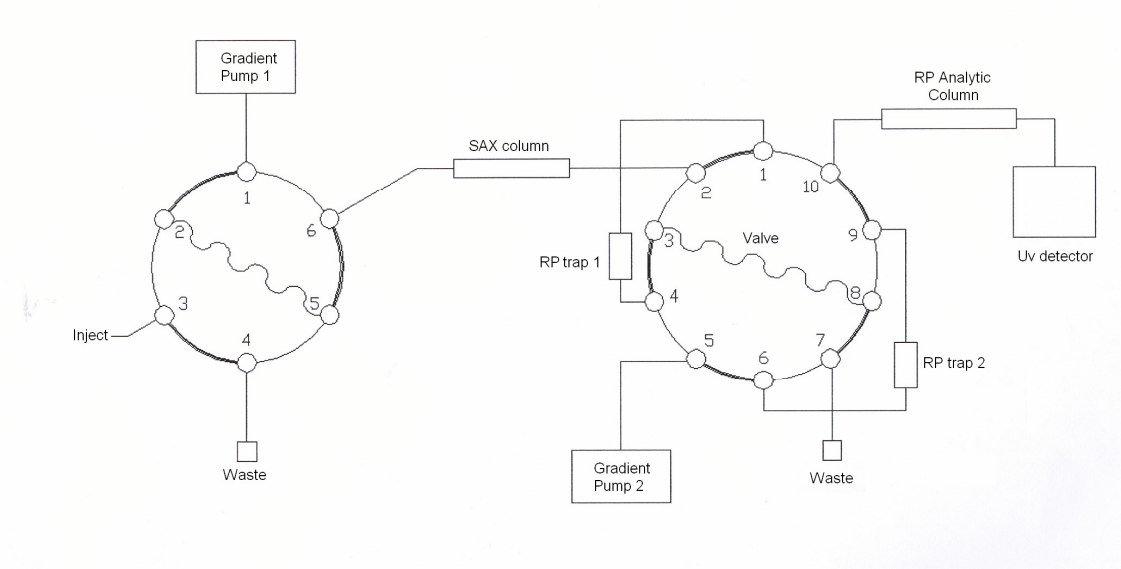

Figure 1: Principle for on-line trapping. The proteins are first separated according to pI value on the SAX column. As the proteins are eluted from the SAX column, they are trapped on RP trap 1. Fraction 1 consists of all proteins eluted during the trapping time. After a given time (trapping time), the valve is switched. The proteins eluting from the SAX after switching are trapped on RP trap column 2. Meanwhile, the proteins on RP trap 1 are backflushed using gradient pump 2 and transported to the RP analytical column for separation.

4. **Results and discussion:**

The SAX column did not have enough resolving power to completely separate the plasma proteins. The anion exchange chromatography separation (fig 2) shows several peaks containing multiple protein species. The pH was measured off-line. An on-line measuring procedure was also considered and tested, but rejected. The flow rate of 1 ml/min seemed too fast to allow satisfactory measurement of pH (Appendix F).

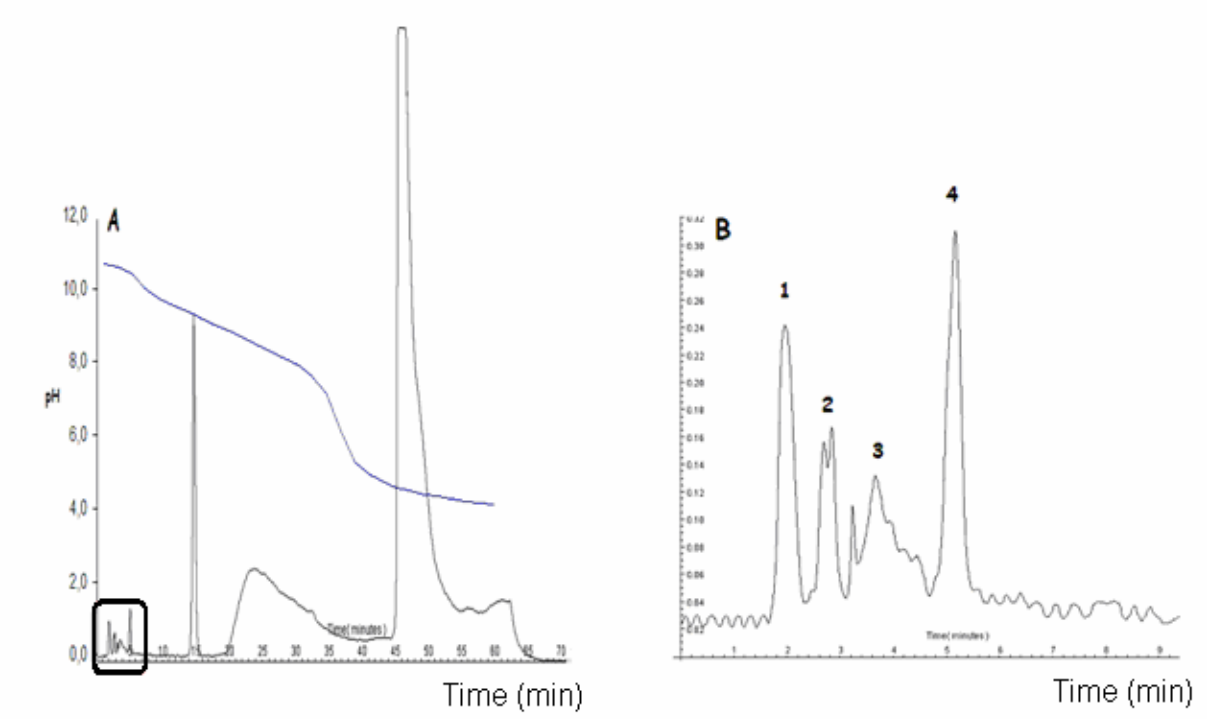

Figure 2A-B: Chromatogram of plasma proteins (1 ml 1+5diluted) on SAX column. The pH profile is superimposed in chromatogram A. A gradient running from 100% buffer A (pH 11.2) to 100% Buffer B (pH 3.4) was used. The system remained at 100% B for 30 minutes to ensure complete elution of the sample before returning to 100% A. The basic proteins of interest are located to the left in chromatogram 2A. 2B shows this area expanded. A strong anion exchange column, 8 µm particles, 1000 Å pore size 150x 4.6 mm column was used. The mobile phase consisted of 0.100 M diethanolamine/ethanoleamine/piperazine buffer. The proteins were detected with UV-detection at 280 nm. The flow rate was 1 ml/min.

The chromatogram of the basic fraction (figure 2B) shows four main peaks (1-4), each probably containing multiple proteins. By trapping the peaks one by one, using the switching device and trap columns, separation could be further improved in the second dimension.

This system only has the ability to trap two fractions. If a four fraction trapping is to be carried out on this system, it has to be performed in two steps, involving two stages of plasma injections: First, the material eluted from the SAX in 0-2.5 minutes is trapped on trap column 1. Then, the valve is switched and the first fraction is transferred to the analytical column while the second fraction eluting from 2.5 – 3 minutes is trapped on the second trap column. Since the system has no further positions available, the remaining plasma can not be separated at this stage, and has to be wasted by manually detaching the tubing from the SAX column. When the first fraction has been completely separated on the analytical column, the valve is switched again to transfer the second fraction to the analytical column. Separating the third and fourth fraction (3-4.5 min and 4.5-6 min) can be achieved in a similar way, by injecting a new plasma sample with the tubing between the SAX and switching device being detached for the first three minutes and reattach to collect the fractions of interest.

With a more advanced system containing several more traps, the procedure could be achieved by only one injection. An additional switching device placed between the first column and the valve could allow a more convenient way of wasting redundant material.

Fractionating is a time and labour consuming process which should be avoided unless it is required to complete a satisfactory separation. The degree of fractionation depends on the separating power of the second dimension column. Therefore, it was first investigated if the second dimension could provide an acceptable separation of the entire basic fraction (fig 2B) without any prior fractionations. If this is the case, switching and trapping would be a waste of time and a pointless complication of the separation process.

The trapping time was first selected to be 10 minutes to collect the entire basic fraction, and trapping was only carried out on trap column 1. After switching, the entire basic fraction was transferred to the analytical column.

However, this on-line switching experiment failed. A broad interfering peak occurred when the valve was switched during a blank run. An additional test where sample material was injected under the same conditions was also performed to see if the interfering peak would overlap the protein peaks. No additional peaks were observed compared with the blank run. Several experiments were carried out to identify the source of this peak, by stripping the system and performing runs while connecting the components one by one (Figure 3). Initially,

water was used as mobile phase in pump 1 and 95% Acn + 0.1% TFA was used as mobile phase for pump 2.

First, all columns were detached (fig 3A). No peaks or irregularities were observed running the gradients. Subsequently, the trap columns (fig 3B) and then the analytical column (fig 3C) were attached. The trap columns were indicated as the source of the peak. The interferences only appeared if any mobile phase from the first dimension system had passed through the trap column. By attaching the analytical column, the retention time of the peak increased, indicating interactions with the reversed phase column. By reducing the amount of acetonitrile in mobile phase 2 from 95% to 5% (fig 3D), the peak disappeared, possibly due to full retention on the analytical column. When a pump 2 mobile phase gradient run from 0-80% B in 30 minutes was performed, (fig 3E) the peak reappeared, earlier and narrower than in the case of the isocratic 95% acetonitrile mobile phase. When the mobile phase in pump 1 was replaced with the salt buffer (pH 11.1), the peak became broader, more intense, and took on a more irregular shape (3F).

The material causing the peaks was not identified. The peaks appeared during a blank run when neither the fraction nor the analytical column were attached to the system, excluding these from being the source, although it seems that the analytical column in some way affects the interfering material.

The fact that the peaks only appeared when any mobile phase from pump 1 had passed through the trap column prior to switching indicates that the mobile phase could bee the source. On the other hand, peaks also appeared when the first dimension mobile phase was pure water. Water should not provide such an intense signal when being detected at 280 nm. The reservoir containing the water was properly cleaned and the whole system was flushed with the water before conducting the experiment, so no pollutants should theoretically be presented.

No notable differences were observed for the different switching position, excluding the possibility that one of the trap columns had been contaminated by plasma leftovers or other materials. There is of course a possibility that they both had been contaminated the same way.

A third possibility is impurities like rust or contaminated material inside the switching devices. To minimize this possibility, both switching devices were opened, checked and cleaned before performing these experiments. No contaminating material was observed.

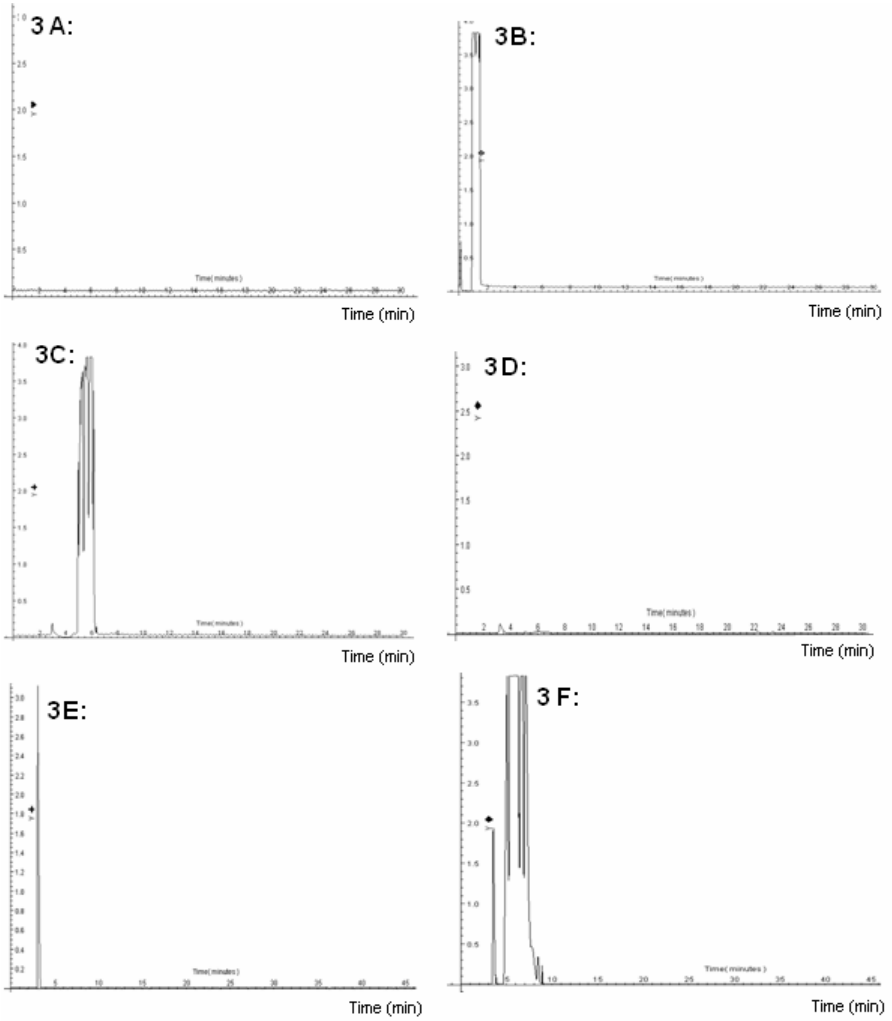

Figure 3 A-F: Chromatograms obtained when searching for interfering peak. ***A:*** *All columns including the trap columns were detached. Mobile phase from pump 1 was type I water, mobile phase in pump 2 (to detector) was Acn /water/TFA 95%/5%/0.1% (volume). Monitoring started when switching was initiated. Flow rate for system 1 was 1 ml/min. Flow rate for system 2 was 0.5 ml/min.* ***B:*** *The procedure was repeated with trap columns attached, and pump 1 mobile phase was flushed through the traps before switching.* ***C:*** *All columns attached.* ***D:*** *The same conditions as in C, but the purified water in pump 2 was replaced with Acn/water/TFA 5% /95%/0.1% (volume).* ***E:*** *A mobile phase gradient for pump 2 was performed, running from 5 to 95% Acn in 30 minutes and remained at 95% Acn for10 minutes.* ***F:*** *The gradient from E was repeated, but now, the mobile phase in pump 1 was replaced with salt buffer (experimental part) pH 11.1. All experiments A-E were carried out with 4 replicates, 2 in each switching position. No considerable differences were observed between the trap columns (switch positions). Replicate peaks in experiment B, C, E and, F did not look 100% identical as for shape, intensity, and retention time. A random chromatogram is presented here.*

To overcome the interference problem, an alternative off-line procedure was developed. When being separated in the analytical column, no interferences were observed in a blank sample performed by trapping three fractions of purified water on the trap column and following the same procedures as the sample.

As seen in figure 4, a complete separation of the proteins in the entire basic fraction of the plasma sample was not achieved. By splitting the basic fraction into smaller fractions as discussed above, complete separation could probably be achieved. This was never performed, however, since the off-line method was a very time consuming procedure and since the purpose of this study was to achieve a time saving on-line separation. Spending weeks on finding the optimized conditions to complete the separation off-line was considered waste of time.

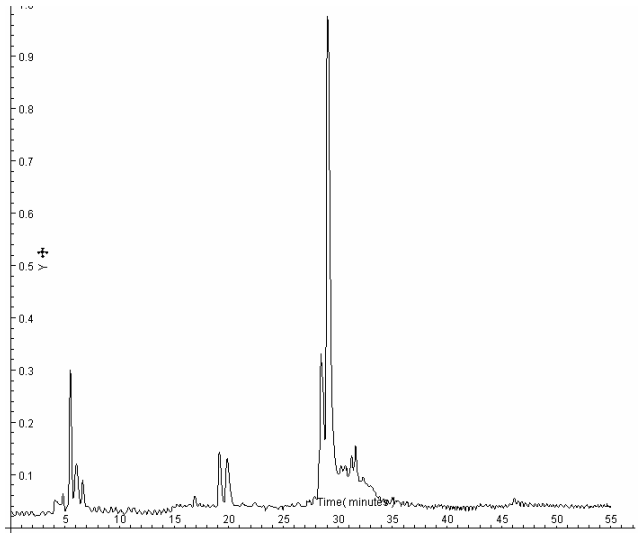

Figure 4: Ttwo-dimensional off-line separation of the basic fraction. The chromatogram shows the second dimension. The first dimension separation procedure was the same as described in figure 2. After 10 minutes and complete trapping of the basic fraction, the eluent from the SAX column was led to waste for complete elution of the remaining proteins with 100% A in 30 minutes before repeating the procedure three times. After trapping the third basic fraction, it was eluted using Acn /water/TFA (85/15/0.1 v/v) at a flow of 1 ml/min and collected in a polypropylene vial. Second dimension separation was carried out using a gradient running from 0-80% B in 30 minutes, at a flow rate of 0.5 ml/min. The column was a 150 x 4.6 mm PLRS-S column with 3 µm particles and 300 Å pore size. The proteins were detected with UV-detection at 280 nm.

5. Conclusions:

The on-line system failed, because of a large interfering peak. The source of the peak was not directly identified, but it seems that the trap columns played an important part.
Further works on this project should focus on testing alternative trap columns.
As an alternative to the on-line separation, an off-line separation method was developed and tested. It turned out to become a very labour and time consuming procedure involving several manual steps, and was only tested once trapping only one large basic fraction. The second dimension was not able to complete the separation of the selected fraction. An important reason for developing a separation based on liquid chromatography is to save time and avoid manual steps. The off-line method that was developed is not a suitable option for this purpose, and no further tests were performed.

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7. Appendix:

A. Data from 4.1:

Table A1a-e: Obtained raw data.

A1a: Cytochrome- C

Replicate	Temperature (ºC)	Retention time (min)	Area
I	25 (ref)	36.050	65946
II	25 (ref)	35.450	72747
III	25 (ref)	35.283	79439
	Average	**35.594**	**72711**
	sd	0.403	6747
	sd %	1.13	9.28
I	50	30.767	67325
II	50	30.700	75416
III	50	30.667	80767
	Average	**30.711**	**74503**
	sd	0.051	6767
	sd %	0.17	9.08
I	75	24.117	76318
II	75	24.100	77766
III	75	24.400	59682
	Average	**24.206**	**71255**
	sd	0.169	10049
	sd %	0.70	14.10
I	100	21.367	27108
II	100	20.967	25151
III	100	20.850	25861
	Average	**21.061**	**26040**
	sd	0.271	991
	sd %	1.29	3.80

A1b: Myoglobin

Replicate	Temperature (ºC)	Retention time (min)	Area
I	25 (ref)	60.183	93477
II	25 (ref)	59.500	98519
III	25 (ref)	59.233	103169
Average		**59.639**	**98388**
sd		0.490	4847
sd %		0.82	4.93
I	50	55.133	96372
II	50	54.950	104213
III	50	55.000	88263
Average		**55.028**	**96283**
sd		0.095	7975
sd %		0.17	8.28
I	75	45.700	90823
II	75	45.833	88764
III	75	46.117	80977
Average		**45.883**	**86855**
sd		0.213	5193
sd %		0.464	5.98
I	100	39.550	116537 outlayer
II	100	39.433	58084
III	100	39.217	57912
Average		**39.400**	**57998**
sd		0.169	122
sd %		0.43	0.21

A1c: Lysozyme

Replicate	Temperature (ºC)	Retention time (min)	Area
I	25 (ref)	43.550	80267
II	25 (ref)	43.033	72158
III	25 (ref)	42.900	76112
	Average	**43.161**	**76179**
	Sd	0.343	4055
	Sd %	0.80	5.32
I	50	37.850	100037
II	50	37.667	104213
III	50	37.750	94564
	Average	**37.756**	**99605**
	Sd	0.092	4839
	Sd %	0.24	4.86
I	75	30.083	100597
II	75	30.067	99795
III	75	30.333	87347
	Average	**30.161**	**95913**
	Sd	0.149	7429
	Sd %	0.49	7.75
I	100	25.283	47188
II	100	25.150	58084
III	100	25.05	58550
	Average	**25.161**	**54607**
	Sd	0.117	6430
	Sd %	0.46	11.77

A1d: Albumin bovine

Replicate	Temprature (ºC)	Retention time (min)	Area
I	25	8.700	135894
II	25	8.700	148561
III	25	8.750	149453
Average		8.717	144636
Sd		0.029	7584
Sd %		0.33	5.24
I	50	8.417	139887
II	50	8.383	132064
III	50	8.433	116520
Average		8.411	129490
Sd		0.03	11894
Sd %		0.30	9.19
I	75	7.333	118518
II	75	7.383	119032
III	75	7.400	123822
Average		7.372	120457
Sd		0.03	2925
Sd %		0.47	2.43
I	100	5.583	118114
II	100	5.600	123761
III	100	5.550	124394
Average		5.578	122090
Sd		0.025	3458
Sd %		0.46	2.83

A1e: Apoferritin

Replicate	Temperature (ºC)	Retention time (min)	Area
I	25 (ref)	12.500	102911
II	25 (ref)	12.367	112163
III	25 (ref)	12.500	124455
	Average	12.456	113176
	Sd	0.077	10808
	Sd %	0.62	9.55
I	50	12.150	123545
II	50	12.200	110634
III	50	12.233	94348
	Average	12.194	109509
	Sd	0.042	14631
	Sd %	0.34	13.36
I	75	10.717	76174
II	75	10.783	78449
III	75	10.583	85352
	Average	10.694	79992
	Sd	0.102	4780
	Sd %	0.95	5.98
I	100	8.417	26027
II	100	8.150	49420
III	100	8.050	55538
	Average	8.206	43662
	Sd	0.190	15575
	Sd %	2.31	35.67

Table A2a: Relative peak area of five different proteins as function of temperature. Peak area at 25ºC is used as reference. N means no significant difference due to reference when variance within and between replicates are compared. L means significantly lower than reference according to a 95% confidence interval, two-tailed ANOVA test where n=3.

Temperature (ºC)	Cytochrome-C		Lysozyme		Myoglobin		Albumin bovine		Apoferritin	
25 (ref)	**100**	**-**	100	-	100	-	100	-	100	-
50	100	N	130	N	100	N	89	N	96	N
75	96	N	125	N	90	N	83	N	70	N
100	35	L	71	N	60	L	84	N	38	N

Table A2b: Relative retention of five different proteins as function of temperature. Peak area at 25ºC is used as reference. N means no significant difference due to reference when variance within and between replicates are compared. L means significantly lower than reference according to a 95% confidence interval, two-tailed ANOVA test where n=3.

Temperature (ºC)	Cytochrome- C		Lysozyme		Myoglobin		Albumine bovine		Apoferritin	
25 (ref)	**100**	**-**	100	-	100	-	100	-	100	-
50	86	L	87	L	92	L	96	L	97	N
75	68	L	69	L	76	L	84	L	85	L
100	59	L	58	L	65	L	63	L	65	L

B. Data from 4.2:

Table B1a-e: obtained raw data

B1a: Cytochrome-C

Replicate	Temperature (ºC)	Retention time (min)	Area
I	ref	20.767	58993
II	ref	20.733	64035
III	ref	20.683	82925
	Average	**20.728**	**68651**
	Sd	0.042	12616
	Sd %	0.20	18.38
I	50	20.683	62595
II	50	20.600	67625
III	50	20.467	70631
	Average	**20.583**	**66950**
	Sd	0.11	4060
	Sd %	0.529	6.06
I	75	20.533	83763
II	75	20.633	71546
III	75	20.683	68930
	Average	**20.616**	**74746**
	Sd	0.076	7917
	Sd %	0.37	10.59
I	100	21.250	16952
II	100	-	-
III	100	-	-
	Average	21.250	16952
	Sd	-	-
	Sd %	-	-
I	50 cool	28.233	65072
II	50 cool	28.267	62707
III	50 cool	28.283	73208
	Average	**28.261**	**66996**
	Sd	0.026	5508
	Sd %	0.09	8.22
I	75 cool	**28.567**	**67885**
II	75 cool	28.550	72102
III	75 cool	28.650	65619
	Average	28.589	68535
	Sd	0.054	3290
	Sd %	0.19	4.80
I	100 cool	-	0
II	100 cool	-	0
III	100 cool	-	0
	Average	-	-
	Sd	-	-
	Sd %	-	-

B1b: Myoglobin

Replicate	Temperature (ºC)	Retention time (min)	Area
I	ref	34.750	86193
II	ref	34.683	90217
III	ref	34.700	91983
	Average	34.711	89464
	Sd	0.035	2967
	Sd %	0.10	3.32
I	50	34.667	83036
II	50	34.617	84222
III	50	34.733	83761
	Average	34.672	83673
	Sd	0.058	598
	Sd %	0.168	0.715
I	75	34.733	49026
II	75	34.733	29092
III	75	34.750	38297
	Average	34.739	38805
	Sd	0.010	9977
	Sd %	0.03	25.71
I	100	34.667	6792
II	100	34.650	4937
III	100	34.533	9439
	Average	34.617	7056
	Sd	0.073	2263
	Sd %	0.21	32.07
I	50 cool	34.657	89092
II	50 cool	34.650	85509
III	50 cool	34.650	84652
	Average	34.652	86418
	Sd	0.004	2355
	Sd %	0.012	2.726
I	75 cool	34.683	34891
II	75 cool	34.650	17665
III	75 cool	34.650	23855
	Average	34.661	25470
	Sd	0.019	8726
	Sd %	0.05	34.26
I	100 cool	34.719	16410
II	100 cool	33.650	11999
III	100 cool	-	-
	Average	34.185	14205
	Sd	0.756	3119
	Sd %	2.211	21.958

B1c: Lysozyme

Sample	Temperature (ºC)	Retention time (min)	Area
I	ref	28.417	67804
II	ref	28.400	77202
III	ref	28.433	95970
	Average	28.417	80325
	Sd	0.017	14340
	Sd %	0.06	17.85
I	50	28.350	97773
II	50	28.200	117585
III	50	28.350	101051
	Average	28.300	105470
	Sd	0.087	10619
	Sd %	0.31	10.07
I	75	28.767	36845 outlayer
II	75	28.700	19977
III	75	28.917	20169
	Average	28.795	20073
	Sd	0.111	136
	Sd %	0.39	0.68
I	100	-	-
II	100	-	-
III	100	28.950	8523
	Average	28.950	8523
	Sd	-	-
	Sd %	-	-
I	50 cool	28.233	87802
II	50 cool	28.267	98234
III	50 cool	28.283	90160
	Average	28.261	92065
	Sd	0.026	5471
	Sd %	0.09	5.94
I	75 cool	28.567	58159 outlayer
II	75 cool	28.550	29404
III	75 cool	28.650	17379
	Average	28.589	23392
	Sd	0.054	8503
	Sd %	0.19	36.35
I	100 cool	-	0
II	100 cool	-	0
III	100 cool	-	0
	Average	-	0
	Sd	-	0
	Sd %	-	-

B1d: Albumin bovine

Sample	Temperature (ºC)	Retention time (min)	Area
I	Ref	6.267	148284
II	Ref	6.250	154874
III	Ref	6.233	124056
	Average	6.250	142405
	Sd	0.017	16228
	Sd %	0.27	11.40
I	50	6.283	141514
II	50	6.233	136552
III	50	6.233	139418
	Average	6.250	139161
	Sd	0.029	2491
	Sd %	0.46	1.79
I	75	6.300	49894
II	75	6.317	38759
III	75	6.300	36242
	Average	6.306	41632
	Sd	0.010	7265
	Sd %	0.16	17.45
I	100	6.383	4285
II	100	6.317	3702
III	100	6.250	6240
	Average	6.317	4742
	Sd	0.067	1329
	Sd %	1.05	28.03
I	50 cool	6.133	122470
II	50 cool	6.150	128875
III	50 cool	6.150	131777
	Average	6.144	127707
	Sd	0.010	4762
	Sd %	0.16	3.73
I	75 Cool	6.133	58127
II	75 Cool	6.183	39018
III	75 Cool	6.283	31671
	Average	6.200	42939
	Sd	0.076	13657
	Sd %	1.23	31.81
I	100 Cool	6.217	5433
II	100 Cool	6.083	4482
III	100 Cool	6.150	2353
	Average	6.150	4089
	Sd	0.067	1577
	Sd %	1.09	38.57

B1e: Apoferritin

Replicate	Temperature	Retention time (min)	Area
I	ref	10.100	131292
II	ref	10.083	131675
III	ref	10.083	136338
	Average	10.089	133102
	Sd	0.010	2809
	Sd %	0.10	2.11
I	50	10.067	127714
II	50	10.067	116741
III	50	10.033	121164
	Average	10.056	121873
	Sd	0.020	5521
	Sd %	0.20	4.53
I	75	10.017	178825
II	75	10.000	196286
III	75	10.000	183489
	Average	10.006	186200
	Sd	0.010	9041
	Sd %	0.10	4.86
I	100	9.883	245922
II	100	9.733	282981
III	100	9.960	320451
	Average	9.859	283118
	Sd	0.115	37265
	Sd %	1.17	13.16
I	50 cool	10.000	107932
II	50 cool	9.983	112298
III	50 cool	9.933	111976
	Average	9.972	110735
	Sd	0.035	2433
	Sd %	0.35	2.20
I	75 cool	9.900	161604
II	75 cool	9.867	178495
III	75 cool	9.867	192136
	Average	9.878	177412
	Sd	0.019	15295
	Sd %	0.19	8.62
I	100 cool	9.550	247347
II	100 cool	9.583	289151
III	100 cool	9.550	276626
	Average	9.561	271041
	Sd	0.019	21454
	Sd %	0.20	7.92

Table B2: Relative peak areas of five different proteins after being heated for one hour at 50ºC, 75ºC, or 100ºC. Non heated samples are used as reference. For cooled samples, the peak area is compared with the respective peak area right after heating. N means no significant difference due to the reference, L means significantly lower than the reference, and H means significantly higher than the reference according to a 95% confidence interval, two-tailed ANOVA test where n=3.

Temperature (ºC)	Cytochrome-C		Lysozyme		Myoglobin		Albumine Bovine		Apoferritin	
25 ref	100	-	100	-	100	-	100	-	100	-
50	97	N	131	N	93	N	97	N	91	N
75	108	N	24	L	43	L	29	L	139	H
100	24	L	10	L	7	L	3	L	212	H
50 Cooling	100	N	114	N	103	N	91	N	90	N
75 Cooling	91	N	29	N	65	N	103	N	95	N
100 Cooling	0	-	0	-	201	L	86	N	95	N

C. Data from 4.3:

C1a-e: Obtained raw data.

C1a: Cytochrome-C

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	25 (ref)	0	4.333	63219
II	25 (ref)	0	3.917	75721
III	25 (ref)	0	3.950	66961
		Average	4.067	68634
		Sd	0.231	6417
		Sd %	5.69	9.35
I	25	5	6.217	69129
II	25	5	6.183	77614
III	25	5	6.167	78974
		Average	6.189	75239
		Sd	0.03	5335
		Sd %	0.41	7.09
I	25	10	6.183	81500
II	25	10	6.217	85413
III	25	10	6.250	83968
		Average	6.217	83627
		Sd	0.034	1979
		Sd %	0.54	2.37
I	25	30	7.817	68412
II	25	30	7.817	73836
III	25	30	7.850	77714
		Average	7.828	73321
		Sd	0.019	4672
		Sd %	0.24	6.37
I	25	60	7.883	68289
II	25	60	7.533	66602
III	25	60	7.600	69889
		Average	7.672	68260
		Sd	0.186	1644
		Sd %	2.42	2.41
I	50 (ref)	0	3.700	77449
II	50 (ref)	0	3.800	82937
III	50 (ref)	0	3.750	78384
		Average	3.750	79590
		Sd	0.050	2936
		Sd %	1.33	3.69
I	50	5	6.567	78559
II	50	5	6.533	82144
III	50	5	6.517	83097
		Average	6.539	81267
		Sd	0.026	2393
		Sd %	0.39	2.94

C1b: Myoglobin

Replicate	Temperature(ºC)	Delay (min)	Retention (min)	Area
I	25 ref	0	9.800	101892
II	25 ref	0	9.750	102078
III	25 ref	0	9.733	105291
		Average	**9.761**	**103087**
		Sd	0.03	1911
		Sd %	0.36	1.85
I	25	5	9.850	100096
II	25	5	9.817	111905
III	25	5	9.833	104355
		Average	**9.833**	**105452**
		Sd	0.017	5980
		Sd %	0.17	5.67
I	25	10	9.857	102704
II	25	10	9.883	105594
III	25	10	9.883	105922
		Average	**9.874**	**104740**
		Sd	0.015	1771
		Sd %	0.15	1.69
I	25	30	12.233	86834
II	25	30	12.200	105858
III	25	30	12.233	101705
		Average	**12.222**	**98132**
		Sd	0.019	10003
		Sd %	0.16	10.19
I	25	60	12.150	100295
II	25	60	12.117	97659
III	25	60	12.200	108014
		Average	**12.156**	**101989**
		Sd	0.042	5381
		Sd %	0.34	5.28
I	50 ref	0	15.133	110179
II	50 ref	0	15.167	114072
III	50 ref	0	15.133	115925
		Average	**15.144**	**113392**
		Sd	0.020	2933
		Sd %	0.13	2.59
I	50	5	15.267	129662
II	50	5	15.333	134072
III	50	5	15.300	133787
		Average	**15.3**	**132507**
		Sd	0. 033	2468
		Sd %	0.22	1.86

Table C1.a-b continued:

C1a: Cytochrome-C

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	50	10	6.500	85158
II	50	10	6.550	83000
III	50	10	6.550	79264
		Average	6.533	82474
		Sd	0.029	2982
		Sd %	0.44	3.62
I	50	30	6.550	73939
II	50	30	6.600	83589
III	50	30	6.567	83537
		Average	6.572	80355
		Sd	0.025	5556
		Sd %	0.39	6.91
I	50	60	6.450	81875
II	50	60	6.467	76317
III	50	60	6.517	75852
		Average	6.478	78015
		Sd	0.035	3351
		Sd %	0.54	4.30
I	75 (ref)	0	3.567	65072
II	75 (ref)	0	3.483	75042
III	75 (ref)	0	3.467	77385
		Average	3.506	72500
		Sd	0.054	6538
		Sd %	1.53	9.02
I	75	5	6.433	69424
II	75	5	6.417	74527
III	75	5	6.400	73466
		Average	6.417	72472
		Sd	0.017	2693
		Sd %	0.26	3.72
I	75	10	6.450	79102
II	75	10	9.017 outlayer	68941
III	75	10	6.467	82664
		Average	6.459	76902
		Sd	0.012	7121
		Sd %	0.19	9.26
I	75	30	6.467	69209
II	75	30	6.433	78701
III	75	30	6.400	69290
		Average	6.433	72400
		Sd	0.034	5457
		Sd %	0.52	7.54

C1b: Myoglobin

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	50	10	15.267	135011
II	50	10	15.283	126410
III	50	10	15.300	125871
		Average	15.283	129097
		Sd	0.017	5128
		Sd %	0.11	3.97
I	50	30	16.300	126852
II	50	30	16.267	124106
III	50	30	15.267	132468
		Average	15.945	127809
		Sd	0.587	4262
		Sd %	3.68	3.33
I	50	60	15.217	128670
II	50	60	15.233	127773
III	50	60	15.267	126224
		Average	15.239	127556
		Sd	0.026	1237
		Sd %	0.17	0.97
I	75 ref	0	12.067	96522
II	75 ref	0	12.100	113402
III	75 ref	0	12.050	107327
		Average	12.072	105750
		Sd	0.025	8550
		Sd %	0.21	8.08
I	75	5	12.183	127573
II	75	5	12.133	131499
III	75	5	12.150	131468
		Average	12.155	130180
		Sd	0.025	2258
		Sd %	0.21	1.73
I	75	10	12.167	125190
II	75	10	12.223	131551
III	75	10	12.183	131563
		Average	12.191	129435
		Sd	0.029	3676
		Sd %	0.24	2.84
I	75	30	12.200	132921
II	75	30	12.167	139795
III	75	30	12.200	131692
		Average	12.189	134803
		Sd	0019	4367
		Sd %	0.16	3.24

Table 1C. .a-b continued:

1Ca: Cytochrome-C

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	75	60	6.283	69735
II	75	60	6.467	51984
III	75	60	6.467	65392
		Average	6.406	62370
		Sd	0.106	9253
		Sd %	1.66	14.84
I	100 (ref)	0	5.867	62767
II	100 (ref)	0	6.100	65153
III	100 (ref)	0	5.483	64453
		Average	5.817	64124
		Sd	0.312	1226
		Sd %	5.36	1.91
I	100	5	8.383	45191
II	100	5	8.267	46495
III	100	5	8.617	54994
		Average	8.422	48893
		Sd	0.178	5323
		Sd %	2.12	10.89
I	100	10	8.250	49036
II	100	10	8.350	38160
III	100	10	8.300	52228
		Average	8.300	46475
		Sd	0.050	7375
		Sd %	0.60	15.87
I	100	30	8.400	24454
II	100	30	8.367	40260
III	100	30	8.250	32830
		Average	8.339	32515
		Sd	0.079	7908
		Sd %	0.95	24.32
I	100	60	8.733	24880
II	100	60	8.700	13315
III	100	60	8.700	14202
		Average	8.711	17466
		Sd	0.019	6436
		Sd %	0.22	36.85

1Cb: Myoglobin

Replicate	Temperature(ºC)	Delay (min)	Retention (min)	Area
I	75	60	12.150	126401
II	75	60	12.167	113541
III	75	60	12.183	134196
		Average	12.167	124713
		Sd	0.017	10430
		Sd %	0.14	8.36
I	100 ref	0	15.700	95432
II	100 ref	0	15.733	92627
III	100 ref	0	15.733	92114
		Average	15.722	93391
		Sd	0.019	1786
		Sd %	0.12	1.91
I	100	5	15.717	98157
II	100	5	15.783	108212
III	100	5	15.717	102139
		Average	15.739	102836
		Sd	0.038	5064
		Sd %	0.24	4.92
I	100	10	15.700	101497
II	100	10	15.717	83638
III	100	10	15.700	92344
		Average	15.706	92493
		Sd	0.010	8930
		Sd %	0.06	9.66
I	100	30	15.683	85451
II	100	30	15.717	90272
III	100	30	15.717	84783
		Average	15.706	86835
		Sd	0.020	2995
		Sd %	0.12	3.45
I	100	60	15.750	79025
II	100	60	15.750	84897
III	100	60	15.683	82053
		Average	15.728	81992
		Sd	0.039	2936
		Sd %	0.246	3.58

Table 1C. c-d:

1Cc: Lysozyme

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	25 ref	0	7.817	79983
II	25 ref	0	7.783	96484
III	25 ref	0	7.833	89391
		Average	**7.811**	**88619**
		Sd	0.03	8278
		Sd %	0.33	9.34
I	25	5	7.917	106175
II	25	5	7.917	106085
III	25	5	7.917	111456
		Average	**7917**	**107905**
		Sd	1.088E-15	3075
		Sd %	0.00	0.00
I	25	10	7933	109374
II	25	10	7.917	103482
III	25	10	7.950	108430
		Average	**7.933**	**107095**
		Sd	0.02	3165
		Sd %	0.21	2.95
I	25	30	10.217	102414
II	25	30	10.217	107380
III	25	30	10.267	86547
		Average	**10.234**	**98780**
		Sd	0.029	10881
		Sd %	0.28	11.02
I	25	60	10.250	104175
II	25	60	10.183	103080
III	25	60	10.150	112246
		Average	**10.194**	**106500**
		Sd	0.051	5006
		Sd %	0.50	4,700
I	50 ref	0	8.500	89606
II	50 ref	0	8.550	95141
III	50 ref	0	8.567	96143
		Average	**8.539**	**93630**
		Sd	0.035	3521
		Sd %	0.41	3.76
I	50	5	9.233	97463
II	50	5	9.217	90046
III	50	5	9.183	89035
		Average	**9.211**	**92181**
		Sd	0.026	4602
		Sd %	0.28	4.99

1Cd: Albumin bovine

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	25 ref	0	6.117	136074
II	25 ref	0	6.050	148248
III	25 ref	0	6.083	152624
		Average	6.083	145649
		Sd	0.034	8576
		Sd %	0.55	5.89
I	25	5	6.150	158513
II	25	5	6.100	159547
III	25	5	6.100	159682
		Average	6.117	159247
		Sd	0.03	640
		Sd %	0.47	0.40
I	25	10	6.200	126421
II	25	10	6.183	145461
III	25	10	6.183	150695
		Average	6.189	140859
		Sd	0.01	12775
		Sd %	0.16	9.07
I	25	30	6.033	130082
II	25	30	6.100	131876
III	25	30	6.117	131495
		Average	6.083	131151
		Sd	0.044	945
		Sd %	0.73	0.72
I	25	60	6.133	122187
II	25	60	6.133	119646
III	25	60	6.183	151760
		Average	6.150	131198
		Sd	0.029	17853
		Sd %	0.47	13.61
I	50 ref	0	8.333	141735
II	50 ref	0	8.350	130178
III	50 ref	0	8.317	131287
		Average	8.333	134400
		Sd	0.017	6376
		Sd %	0.20	4.74
I	50	5	8.367	130404
II	50	5	8.383	127696
III	50	5	8.367	103268
		Average	8.372	120456
		Sd	0.009	14947
		Sd %	0.11	12.41

Table 1C.c-d continued:

1Cc: Lysozyme

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	50	10	9.200	91308
II	50	10	9.250	91183
III	50	10	9.233	92344
		Average	9.228	91612
		Sd	0.025	637
		Sd %	0.28	0.70
I	50	30	9.283	82475
II	50	30	9.300	92221
III	50	30	9.300	87626
		Average	9.294	87441
		Sd	0.010	4876
		Sd %	0.11	5.58
I	50	60	9.133	88205
II	50	60	9.133	87838
III	50	60	9.183	91046
		Average	9.150	89030
		Sd	0.029	1756
		Sd %	0.32	1.97
I	75 ref	0	7.767	81557
II	75 ref	0	7.650	82542
III	75 ref	0	7.617	91262
		Average	7.678	85120
		Sd	0.079	5342
		Sd %	1.03	6.28
I	75	5	8.733	81072
II	75	5	8.683	77075
III	75	5	8.717	83271
		Average	8.711	80473
		Sd	0.026	3141
		Sd %	0.29	3.90
I	75	10	8.867	79935
II	75	10	10.633	76795
III	75	10	8.733	87471
		Average	9.411	81400
		Sd	1.060	5487
		Sd %	11.27	6.74
I	75	30	8.833	74940
II	75	30	8.800	82718
III	75	30	8.800	78434
		Average	8.811	78697
		Sd	0.019	3896
		Sd %	0.22	4.95

1Cd: Albumin bovine

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	50	10	8.417	128888
II	50	10	8.350	136713
III	50	10	8.350	129358
		Average	8.372	131653
		Sd	0.039	4388
		Sd %	0.46	3.33
I	50	30	8.350	137782
II	50	30	8.367	139032
III	50	30	8.385	139485
		Average	8.367	138766
		Sd	0.018	882
		Sd %	0.21	0.64
I	50	60	8.400	116191
II	50	60	8.367	128220
III	50	60	8.383	128107
		Average	8.383	124173
		Sd	0.017	6913
		Sd %	0.20	5.57
I	75 ref	0	7.333	134463
II	75 ref	0	7.267	132911
III	75 ref	0	7.267	131755
		Average	7.289	133043
		Sd	0.038	1359
		Sd %	0.52	1.02
I	75	5	7.233	128467
II	75	5	7.267	115779
III	75	5	7.233	120582
		Average	7.244	121609
		Sd	0.020	6406
		Sd %	0.27	5.27
I	75	10	7.267	116352
II	75	10	7.283	117692
III	75	10	7.250	118835
		Average	7.267	117626
		Sd	0.017	1243
		Sd %	0.23	1.06
I	75	30	7.250	114891
II	75	30	7.233	117100
III	75	30	7.267	119369
		Average	7.250	117120
		Sd	0.017	2239
		Sd %	0.23	1.91

Table 1C. c-d continued:

1Cc: Lysozyme

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	75	60	8.683	73132
II	75	60	8.850	68271
III	75	60	8.750	79683
		Average	8.761	73695
		Sd	0.084	5727
		Sd %	0.96	7.77
I	100 ref	0	12.550	57814
II	100 ref	0	12.700	57181
III	100 ref	0	12.467	64934
		Average	12.572	59976
		Sd	0.118	4305
		Sd %	0.94	7.18
I	100	5	13.433	55755
II	100	5	13.317	61832
III	100	5	13.483	62632
		Average	13.411	60073
		Sd	0.085	3761
		Sd %	0.63	6.26
I	100	10	13.083	52342
II	100	10	13.317	49142
III	100	10	13.217	59067
		Average	13.206	53517
		Sd	0.117	5066
		Sd %	0.889	9.466
I	100	30	13.367	33311
II	100	30	13.400	39816
III	100	30	13.267	34914
		Average	13.345	36014
		Sd	0.069	3389
		Sd %	0.519	9.410
I	100	60	13.983	28859
II	100	60	13.567	22136
III	100	60	13.867	21377
		Average	13.806	24124
		Sd	0.215	4118
		Sd %	1.55	17.07

1Cd: Albumin Bovine

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Area
I	75	60	7.250	134011
II	75	60	7.267	125820
III	75	60	7.217	113193
		Average	7.245	124341
		Sd	0.025	10487
		Sd %	0.35	8.43
I	100 ref	0	5.517	111857
II	100 ref	0	5.533	114029
III	100 ref	0	5.550	115733
		Average	5.533	113873
		Sd	0.017	1943
		Sd %	0.30	1.71
I	100	5	5.583	109440
II	100	5	5.550	122212
III	100	5	5.583	120901
		Average	5.572	118023
		Sd	0.019	7026
		Sd %	0.34	5.95
I	100	10	5.550	96621
II	100	10	5,550	101212
III	100	10	5.517	106284
		Average	5.539	101372
		Sd	0.019	4833
		Sd %	0.34	4.77
I	100	30	5.500	108192
II	100	30	5.567	89674
III	100	30	5.500	85481
		Average	5.522	94449
		Sd	0.039	12085
		Sd %	0.70	12.80
I	100	60	5.450	40828
II	100	60	5.500	67497
III	100	60	5.483	62912
		Average	5.478	57079
		Sd	0.025	14259
		Sd %	0.46	24.98

Table 1Ce:

1Ce: Apoferritin

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Areal
I	25 ref	0	9.850	110373
II	25 ref	0	9.857	123476
III	25 ref	0	9.867	128220
		Average	9.858	120690
		Sd	0.009	9244
		Sd %	0.09	7.66
I	25	5	9.850	124649
II	25	5	9.850	131759
III	25	5	9.850	125432
		Average	9.850	127280
		Sd	0.00	3899
		Sd %	0.00	3.06
I	25	10	9.967	82019
II	25	10	9.967	108552
III	25	10	9.950	120284
		Average	9.961	103618
		Sd	0.010	19604
		Sd %	0.10	18.92
I	25	30	9.783	103440
II	25	30	9.850	97654
III	25	30	9.900	100149
		Average	9.844	100414
		Sd	0.059	2902
		Sd %	0.60	2.89
I	25	60	9.983	80237
II	25	60	9.983	78594
III	25	60	10.000	128310
		Average	9.989	95714
		Sd	0.010	28241
		Sd %	0.10	29.51
I	50 ref	0	12.200	85217
II	50 ref	0	12.200	95165
III	50 ref	0	11.983	114584
		Average	12.128	98322
		Sd	0.125	14936
		Sd %	1.03	15.19
I	50	5	12.150	122132
II	50	5	12.217	116765
III	50	5	12.317	69259
		Average	12.228	102719
		Sd	0.084	29101
		Sd %	0.69	28.33

Table 1Ce continued:

1Ce: Apoferritin

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Areal
I	50	10	12.267	120426
II	50	10	12.017	122014
III	50	10	12.067	120985
		Average	12.117	121142
		Sd	0.132	806
		Sd %	1.09	0.66
I	50	30	12.017	129099
II	50	30	12.050	127846
III	50	30	12.217	126145
		Average	12.095	127697
		Sd	0.107	1483
		Sd %	0.89	1.16
I	50	60	12.083	109543
II	50	60	12.167	122952
III	50	60	12.217	119096
		Average	12.156	117197
		Sd	0.068	6903
		Sd %	0.56	5.89
I	75 ref	0	10.467	65976
II	75 ref	0	10.817	70540
III	75 ref	0	10.650	73553
		Average	10.645	70023
		Sd	0.175	3815
		Sd %	1.64	5.45
I	75	5	10.583	82802
II	75	5	10.483	78418
III	75	5	10.400	75749
		Average	10.489	78990
		Sd	0.092	3561
		Sd %	0.87	4.51
I	75	10	10.433	72422
II	75	10	10.467	67965
III	75	10	10.683	76734
		Average	10.528	72374
		Sd	0.136	4385
		Sd %	1.29	6.06
I	75	30	10.650	71697
II	75	30	10.667	74406
III	75	30	10.467	73048
		Average	10.595	73050
		Sd	0.111	1355
		Sd %	1.05	1.85

Table 1Ce: Continued:

1Ce: Apoferritin

Replicate	Temperature (ºC)	Delay (min)	Retention (min)	Areal
I	75	60	10.683	64659
II	75	60	10.667	65335
III	75	60	10.450	60729
		Average	10.600	63574
		Sd	0.130	2487
		Sd %	1.23	3.91
I	100 ref	0	8.250	54972
II	100 ref	0	8.233	53923
III	100 ref	0	8.033	48313
		Average	8.172	52403
		Sd	0.121	3580
		Sd %	1.48	6.83
I	100	5	8.267	36882
II	100	5	8.267	38195
III	100	5	8.017	36132
		Average	8.184	37070
		Sd	0.144	1044
		Sd %	1.76	2.82
I	100	10	8.250	32797
II	100	10	8.233	38551
III	100	10	8.133	33833
		Average	8.205	35060
		Sd	0.063	3067
		Sd %	0.77	8.75
I	100	30	8.200	32774
II	100	30	8.300	30010
III	100	30	8.217	29613
		Average	8.239	30799
		Sd	0.054	1722
		Sd %	0.65	5.59
I	100	60	8.167	22489
II	100	60	8.050	17094
III	100	60	8.167	17162
		Average	8.128	18915
		Sd	0.068	3095
		Sd %	0.83	16.36

Table 2Ca: The effect of residence time on column at different temperatures for cytochrome-C. N means no significant difference due to the reference, L means significantly lower than the reference, and H means significantly higher than the reference according to a 95% interval two-tailed ANOVA test with n=3.

Temp (ºC)	Delay (min)	Relative area	
25 ref	0	100	-
25	5	109	N
25	10	121	N
25	30	106	N
25	60	99	N
50 ref	0	100	-
50	5	102	N
50	10	103	N
50	30	100	N
50	60	98	N
75 ref	0	100	-
75	5	99	N
75	10	106	N
75	30	99	N
75	60	86	N
100 ref	0	100	-
100	5	76	N
100	10	72	N
100	30	50	L
100	60	21	L

Table 2Cb: The effect of residence time on column at different temperatures for myoglobin. N means no significant difference due to the reference, L means significantly lower than the reference and H means significantly higher than the reference according to a 95% interval two-tailed ANOVA test with n=3.

Temp (ºC)	Delay (min)	Relative area	
25 ref	0	100	-
25	5	102	N
25	10	101	N
25	30	95	N
25	60	98	N
50 ref	0	100	-
50	5	116	H
50	10	113	N
50	30	112	N
50	60	112	H
75 ref	0	100	-
75	5	123	N
75	10	122	N
75	30	127	N
75	60	117	N
100 ref	0	100	-
100	5	110	N
100	10	99	N
100	30	92	N
100	60	87	N

Table 2Cc: Effect of residence time on column at different temperatures for lysozyme. N means no significant difference due to the reference, L means significantly lower than the reference, and H means significantly higher than the reference according to a 95% interval two-tailed ANOVA test with n=3.

Temp (ºC)	Delay (mi n)	Relative area	
25 ref	0	100	-
25	5	121	N
25	10	120	N
25	30	111	N
25	60	120	N
50 ref	0	100	-
50	5	98	N
50	10	97	N
50	30	93	N
50	60	95	N
75 ref	0	100	-
75	5	94	N
75	10	95	N
75	30	92	N
75	60	86	N
100 ref	0	100	-
100	5	100	N
100	10	89	N
100	30	60	L
100	60	40	L

Table 2Cd: Effect of residence time on column at different temperatures for albumin bovine. N means no significant difference due to the reference, L means significantly lower than the reference, and H means significantly higher than the reference according to a 95% interval two-tailed ANOVA test with n=3.

Temp (ºC)	Delay (min)	Relative area	
25 ref	0	100	-
25	5	109	N
25	10	96	N
25	30	90	N
25	60	90	N
50 ref	0	100	-
50	5	89	N
50	10	97	N
50	30	103	N
50	60	92	N
75 ref	0	100	-
75	5	91	N
75	10	88	N
75	30	88	N
75	60	93	N
100 ref	0	100	-
100	5	103	N
100	10	89	N
100	30	82	N
100	60	50	L

Table 2Ce: Effect of residence time on column at different temperatures for apoferritin. N means no significant difference due to the reference, L means significantly lower than the reference, and H means significantly higher than the reference according to a 95% interval two-tailed ANOVA test with n=3.

Temp (ºC)	Delay (min)	Relative area	
25 ref	0	100	-
25	5	105	N
25	10	85	N
25	30	83	N
25	60	79	N
50 ref	0	100	-
50	5	104	N
50	10	123	N
50	30	129	N
50	60	119	N
75 ref	0	100	-
75	5	112	N
75	10	103	N
75	30	104	N
75	60	90	N
100 ref	0	100	-
100	5	70	L
100	10	66	N
100	30	58	L
100	60	36	L

D. Data from 4.4:

Table 4a-e: Raw data.

4ai: Cytochrome-C. Temperature gradient

Replicate	Retention time (min)	Area
I	2.617	67078
II	2.617	69871
III	2.667	75583
Average	2.634	70844
Sd	0.029	4335
Sd %	1.10	6.12

4aii: Cytochrome-C. Mobile phase gradient

Replicate	Retention time (min)	Area
I	6,600	77413
II	6,117	73765
III	6,317	80048
Average	6,345	77075
Sd	0,243	3155
Sd %	3,83	4,09

4bi: Myoglobin. Temperature gradient

Replicate	Retention time (min)	Area
I	10.333	93993
II	10.250	117433
III	10.267	122107
Average	10.283	111178
Sd	0.044	15065
Sd %	0.43	13.55

4bii: Myoglobin. Mobile phase gradient

Replicate	Retention time (min)	Area
I	12,067	93096
II	12,083	99538
III	12,150	95867
Average	12,100	96167
Sd	0,044	3231
Sd %	0,36	3,36

4ci: Lysozyme. Temperature gradient

Replicate	Retention time (min)	Area
I	4,900	77857
II	4,733	77238
III	4,833	78456
Average	4,822	77850
Sd	0,084	609
Sd %	1,74	0,78

4cii: Lysozyme. Mobile phase gradient

Replicate	Retention time (min)	Area
I	10.100	78328
II	10.083	88565
III	10.133	86416
Average	10.105	84436
Sd	0.025	5398
Sd %	0.25	6.39

4di: Albumin bovine. Temperature gradient

Replicate	Retention time (min)	Area
I	2.150	146706
II	2.150	144368
III	2.150	143104
Average	2.150	144726
Sd	0.000	1827
Sd %	0.00	1.26

4dii: Albumin bovine. Mobile phase gradient

Replicate	Retention time (min)	Area
I	5.517	136526
II	5.517	133136
III	5.500	139392
Average	5.511	136351
Sd	0.010	3132
Sd %	0.18	2.30

4ei: Apoferritin. Temperature gradient

Replicate	Retention time (min)	Area
I	6.550	132894
II	6.633	139424
III	6.600	141832
Average	6.594	138050
Sd	0.042	4625
Sd %	0.63	3.35

4eii: Apoferritin. Mobile phase gradient

Replicate	Retention time (min)	Area
I	8.200	121810
II	8.250	125556
III	8.167	133599
Average	8.206	126988
Sd	0.042	6024
Sd %	0.51	4.74

E. Off-line pH measurements:

Table A1: Average pH (n=3) of SAX column mobile phase elution time. Each fraction was collected for 2 minutes (2 ml). A gradient running from 100% buffer A (pH 10.70) to 100% buffer B (pH 3.36) in 30 minutes and kept at 100% B for 30 minutes was used and the total flow was 1 ml/min.

Time (min)	I	II	III	Average	sd	sd%
1	10.8	10.5	10.5	**10.7**	0.21	1.92
3	10.7	10.5	10.5	**10.6**	0.13	1.25
5	10.5	10.4	10.4	**10.4**	0.06	0.53
7	10.0	10.0	10.0	**10.0**	0.03	0.29
9	9.8	9.7	9.7	**9.7**	0.02	0.21
11	9.6	9.5	9.5	**9.5**	0.02	0.22
13	9.4	9.3	9.4	**9.4**	0.03	0.33
15	9.2	9.2	9.1	**9.2**	0.05	0.49
17	9.1	9.0	9.0	**9.0**	0.04	0.45
19	8.9	8.8	8.8	**8.8**	0.04	0.46
21	8.7	8.6	8.6	**8.7**	0.05	0.57
23	8.5	8.4	8.4	**8.5**	0.06	0.72
25	8.3	8.2	8.2	**8.3**	0.06	0.74
27	8.2	8.0	8.0	**8.1**	0.06	0.79
29	8.0	X	7.8	**7.9**	0.11	1.34
31	7.8	7.5	7.5	**7.6**	0.19	2.43
33	7.6	7.0	6.8	**7.1**	0.44	6.14
35	6.8	5.9	5.8	**6.2**	0.52	8.46
37	5.4	5.2	5.2	**5.3**	0.11	2.15
39	5.0	4.9	4.9	**4.9**	0.05	1.00
41	4.8	4.7	4.7	**4.7**	0.02	0.42
43	4.6	4.6	4.5	**4.6**	0.06	1.34
45	4.5	4.5	4.5	**4.5**	0.01	0.26
47	4.3	4.4	4.4	**4.4**	0.04	0.95
49	4.4	4.3	4.3	**4.3**	0.02	0.35
51	4.3	4.3	4.3	**4.3**	0.02	0.49
53	4.2	4.2	4.2	**4.2**	0.05	1.10
55	4,2	4.2	4.1	**4.2**	0.04	0.97
57	4.2	4.1	4.1	**4.1**	0.04	0.88
59	4.1	4.1	4.0	**4.1**	0.06	1.48
					avg sd%:	1.29

F. On and off-line measuring of gradient pH:

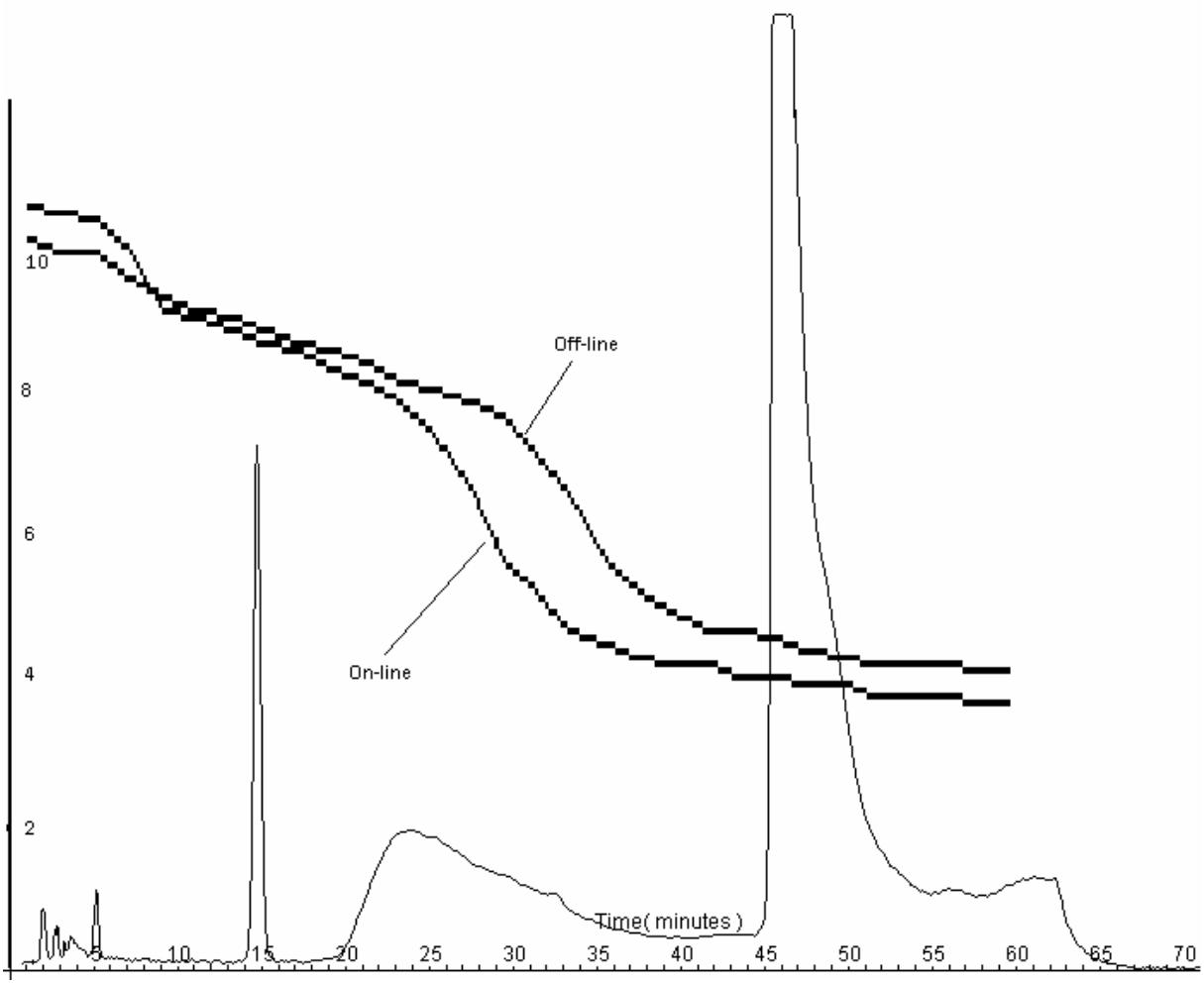

Figure A1: On-line and off-line pH measurement of gradient run plotted versus gradient time. A chromatogram of a plasma sample is shown in the background. Both modes were measured using the same electrode, a round tip double junction flow-through pH electrode. In the on-line mode the signal from the pH electrode was translated with a Cl-6507 pH amplifier and monitored with Science Workshop Data Studio software. In the off-line mode, the mobile phase was collected in vials after eluting from the SAX column, each vial containing 2 ml mobile phase and measured one by one using the same equipment. Both measurements were carried out with three replicates. A random on-line curve is shown. The off-line curve is the average of the replicates (appendix E).