UNIVERSITEIT VAN AMSTERDAM

MSC CHEMISTRY
Analytical Sciences
Master Thesis

Understanding the Elution and Ionization Behavior of Bio-macromolecules in Aqueous SEC-MS

---

by

Iro K. Ventouri

11119667 - 2572868

48 ECTS
September 2016-June 2017

Supervisor

Prof. Dr. Maarten Honing

DSM Resolve

Geleen

The Netherlands

Daily Supervisor

Dr. Rob Haselberg

Division of Bioanalytical Chemistry

Vrije Universiteit Amsterdam

The Netherlands

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

VU
VRIJE UNIVERSITEIT AMSTERDAM
Faculty of Sciences

Internship institute

Internship institute:	Vrije Universiteit Amsterdam
Department:	Department of Chemistry and Pharmaceutical Sciences
Division:	Division of BioAnalytical Chemistry
Address:	De Boelelaan 1085, 1081 HV Amsterdam
Daily Supervisor:	Dr. Rob Haselberg
E-mail address:	r.haselberg@vu.nl
Phone number:	+31205987536

Company Partner

Company partner:	DSM Resolve
Department:	Competence Manager & Principal Scientist
Address:	Urmonderbaan 22, 6167 RD Geleen
Supervisor:	Prof. Dr. Maarten Honing
E-mail address:	Maarten.honing@dsm.com
Phone number:	+31683639515

Personal information

Name:	Iro Konstantina Ventouri
Student number:	UvA - 11119667
	VU - 2572868
E-mail address:	ventouri.iro@gmail.com
Phone number:	+31631080524

September-July

Title: "Understanding the Elution and Ionization Behavior of Bio-macromolecules in Aqueous SEC-MS"

Master Thesis in Chemistry- Track of Analytical Sciences

Iro K. Ventouri, Pierre Lallementstraat 426, Amsterdam, the Netherlands

+31 (0) 631080524

ventouri.iro@gmail.com

UvA-11119667

VU-2572868

University of Amsterdam- Faculty of Science

17-Jul-2017

Examiner: Dr. H. Lingeman   Examiner: Prof.Dr. Govert Somsen

AKNOWLEDGEMENTS

ABSTRACT

The main challenge of coupling size exclusion chromatography (SEC) with mass spectrometry (MS) is the investigation of appropriate compatible mobile phases that are also able to control the non-ideal interactions that govern SEC mechanism. According to present literature the possible appropriate SEC-MS-compatible mobile phase for protein analysis are quite limited (García, 2005). In this study the impact of the mobile phase additives in the protein structure was investigated by using myoglobin as the model protein. Myoglobin was chosen due to the heme group that is non-covalently located in the center of the protein, which enables the detection of alterations in the protein structure, meaning folding or unfolding. The heme group is not only essential for the function of myoglobin but also plays an important role in maintaining the native tertiary structure of the protein (Neya, 1993) and as a result under denaturation and unfolding it is released and easily detectable by MS (m/z 616).

Initially, for the investigation of the elution behavior of biomacromolecules under SEC-UV, the distribution coefficient (K_D) of myoglobin was plotted against the salt concentration at different pHs close to the physiological conditions. Interactions taking place in size exclusion chromatography, namely electrostatic, hydrophobic and hydrogen bonding could be identified. Ionic strength, pH and salt additive can significantly influence the degree of the interactions. However, according to the obtained results with high enough ionic strength almost ideal SEC is possible, as hindrance of the electrostatic interactions between the silanol groups and the analyte can be achieved. The influence of the salt additive in the interactions follows a more complex mechanism in which the hydrogen bonding network and structure of the water are altered. Ammonium formate and especially bicarbonate seem to be less effective in reducing the non-ideal interactions and the reasons for this phenomenon lie in the explanation of the Hofmeister series, surface chemistry and the stability of the salts under the specific conditions, further explanation follows in the experimental section.

Results from Direct infusion experiments in combination with SEC-MS experiments were performed in order to monitor the protein denaturation and highlight the ideal SEC-MS conditions for native-like protein analysis. From the SEC experiments an observed shifting in the elution volume of the intermediate molecular weight proteins, closer to the exclusion limit indicated that there is a possible shape alteration related to the additive used. Myoglobin was observed to be more susceptible to alterations with regard to the different salts. As a result SEC-MS experiments were performed and it seemed that both the elution and ionization of myoglobin follow almost perfectly the Hofmeister series for the anions. In the gas phase different salts showed different intensities of native and denatured charge state distribution of myoglobin. Ammonium formate and especially bicarbonate seem to enhance the protein unfolding, whereas ammonium acetate leads to lower denaturation of the protein especially at lower salt concentrations and pHs closer to the physiological conditions.

In a nutshell, from the SEC-UV-MS experiments the obtained results were indicating structural differences in the analyte as an effect of the chaotropic and kosmotropic anions used. A new tool for structural protein analysis trapped ion mobility mass spectrometer (TIMS-MS), which was very recently installed in the lab was used in order to investigate these structural differences but also to explore the capabilities of this new instrument. Myoglobin and ubiquitin under the different volatile salts were tested. The results obtained from these experiments initiated a discussion about the very impressive possibilities but also the limitations of this technique.

CONTENTS

ABBREVIATIONS

CD   Circular Dichroism

CE   Capillary Electrophoresis

CCS   Collisional Cross Section

EIM   Extracted Ion Mobilogram

ESI   Electrospray Ionization

HIC   Hydrophobic Chromatography

IEX   Ion Exchange Chromatography

IMMS   Ion Mobility Mass Spectrometry

LALS   Low Angle Light Scattering

MALDI   Matrix Laser Desorption Ionization

MALS   Multi-angle Light Scattering

MS   Mass Spectrometry

NMR   Nuclear Magnetic Resonance

PEO   Poly(ethylene) oxide

pI   Isoelectic point

QToF   Quadrupole time-of-flight

RALS   Right Angle Light Scattering

RI   Refractive Index

SEC   Size Exclusion Chromatography

TIMS   Trapped Ion Mobility

ToF   Time-of-flight

UV   Ultraviolet

VI   Viscosity Index

XRD   X-ray Diffraction

INTRODUCTION

Characterization of bio-macromolecules, such as therapeutic proteins used in pharmaceutical industry play a very important role in order to be able to assess and improve protein-based pharmaceuticals. The biological activity of bio-macromolecules strongly depends on their three dimensional structure, which is strongly correlated with their native state. As a result there is an increasing demand to understand and by extend to analyse bio-macromolecules in conditions that are close to the physiological conditions. For that reason nowadays the development of aqueous separation methodologies are of great interest in order to be able to maintain the native structure of the bio-macromolecules and correlate it with their biological activity.

Size-exclusion chromatography is widely used as a purification and characterization technique for protein mixtures because it provides information related to the molecular weight, size and shape of the analysed proteins and possible aggregation. However, the interactions between the protein and the stationary phase should be manipulated by the mobile phase and additives in order to achieve efficient purification and separation. It is equally important that the conditions used are not imposing alterations on the analyte and can preserve their native structure. As a result a good knowledge and understanding of the main separation mechanism and the properties of both the stationary phase and the mobile phase is highly required in order to achieve optimal results.

Protein characterization requires high performance separation techniques and additional characterization with mass spectrometric analysis. The identification of proteins and the determination of their composition and structure by mass spectrometry is evolving daily and it is heading to become a routine analysis. The coupling of different techniques with mass spectrometry is aiming to provide an insight in order to understand the more complex biochemical processes. From analytical chemistry perspective this means that the complexity of the conditions used for protein analysis is significantly increased, as compatibility between the different techniques should be achieved.

Surprisingly, the literature around aqueous SEC-MS methodologies for protein analysis is quite limited. On the one hand, there is a number of papers focusing on the explanation of size exclusion mechanism and the effect of mobile phase additives (Kopaciewicz & Regnier, 1982) (García, 2005) (Arakawa, 2010) (Fekete, 2014). On the other hand there is also a number of papers (Feng, 1993) (Cassou C. A., 2014) explaining the effect of specific additives on the protein ionization mechanism and protein denaturation. However, it seems that there is a gap in literature related to aqueous SEC-MS for protein characterization with focus on the effect of mobile phase additives on protein denaturation and the suitability of SEC-MS for native protein analysis.
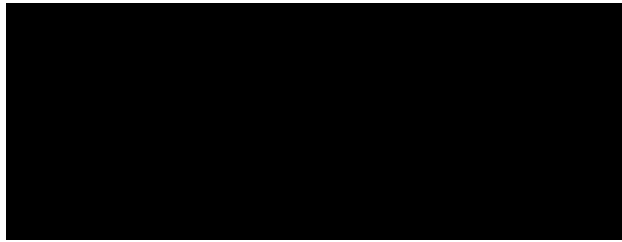
Figure 1 Online detection with SEC-ESI-MS (Wang G. J., 2012).
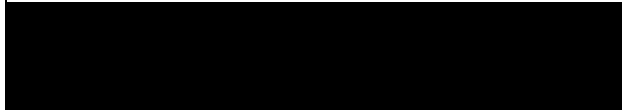
So the main research question and the purpose of this study is to investigate the elution and ionization behavior of biomacromolecules with SEC-ESI-MS, and by extend if these conditions used can ensure native-like protein analysis. The effect of different volatile salts, both kosmotropic and chaotropic according to the Hofmeister series, were investigated under a pH and concentration range close to the physiological conditions.

THEORETICAL BACKGROUND

NATIVE AND DENATURED STATE OF GLOBULAR PROTEINS.

Proteins tend to follow pathways in order to form stable and well defined structures which are known as the native state of a globular protein. The native state of a protein is formed under physiological conditions where the protein is biologically active. This compact conformation contains a low amount of entropy, due to the well-defined structure and intramolecular interactions. Upon folding hydrophobic interactions, hydrogen bonding and van der Waals interactions between the amino acids, provide the native structure of the protein. However, this native conformation is quite susceptible to changes in temperature, extreme pHs, chemicals or interactions with other proteins, which may lead to change of the shape and the inner interactions resulting in activity loss. Under denaturation protein falls into a more open unfolded conformation, with a higher amount of entropy, which is most of the times dysfunctional, but sometimes this is a reversible process. Especially important is that misfolded proteins can aggregate and lead to a variety of diseases, such as Alzheimer's and Parkinson's diseases which are proved to be caused by protein misfolding (Goldberg, 2003).

Many analytical techniques have developed the last decades, which enables native protein analysis. In the field of chromatography aqueous ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and aqueous size exclusion chromatography (SEC) are being used for the characterization of native bio-macromolecules. Mass spectrometry has also becoming a very useful tool for the analysis of proteins at their native structure, either as standalone technique or due to its hyphenation with chromatography. In order to achieve native protein analysis the conditions used should mimic the physiological conditions of the natural environment of the proteins. However, there is a debate about the use of organic solvents in the protein solution and if the use of such conditions should be considered as native protein analysis. Another important requirement for native methodologies is that a wide mass range should be able to be analysed, as proteins can be formulated in multi protein complexes and at the same time smaller or higher molecular weight aggregates co-exist (Vos, 2015).

It is of great importance to be able to monitor possible changes in the higher-order structure of biopharmaceuticals and most importantly to be able to distinguish if structural changes are imposed during analysis, especially because these variations in structure can indicate or even explain differences in the biological or/and immunological response between proteins or between proteins with different PTMs. They can also show differences between proteins of different batches as quality control systems. Despite the progress in the analytical techniques, the characterization of higher-order structure proteins or biopharmaceuticals still remains a challenge, mostly because the in solution dynamics of proteins that are quite complex and involve 'breathing', chain bending, flexing and local unfolding of the structure of the protein.

SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography is a widely used technique for the purification and characterization of biopharmaceutical proteins. The mild mobile phase conditions that are compatible with SEC allow the analysis of proteins causing a minimal impact on their native conformations. Another advantage of SEC is that it can be coupled with a variety of different detectors such as refractive index (RI), ultraviolet (UV), multi-angle light scattering (MALS), viscometer (VI) and with mass spectrometry detectors (MS) (Hong, 2012). SEC can be used under native-like conditions that are actually mimicking the physiological conditions. Most often a pH range close to 7, high salt concentration and 100% aqueous mobile phases are required (Hong, 2012) (Link, 1985).

The separation mechanism in SEC is entropically driven and separates bio-macromolecules according to their hydrodynamic radius. The pore size of the stationary phase plays a very important role in SEC separation, as molecules depending on their size and the pore size will have a different diffusion inside the pores. So the size govern the separation in a higher degree than chemistry. The entropically driven mechanism of SEC, in an ideal occasion that no adsorption occurs (ΔH=0), will form the free-energy equation as follows (Fekete, 2014).

$$\Delta G° = \Delta H - T\Delta S° = RTlnK \qquad \text{Equation 1}$$

$$lnK_d = -\frac{\Delta S°}{R} \qquad \text{Equation 2}$$

$$K_d = \frac{V_r - V_0}{V_i} \qquad \text{Equation 3}$$

In the above mentioned equations ΔG^0, ΔH^0, ΔS^0 is the standard free energy, enthalpy and entropy differences of the chromatographic process. R is the gas constant and T absolute temperature. The factor K_D is the partition coefficient, which is a thermodynamic retention factor and as a result independent from the column dimensions. For the estimation of the K_D factor V_0, which is the void volume, V_R which is the elution volume of the analyte and V_i, which is the intra-particle volume are required. K_D varies from 0 to 1 depending on the diffusion of the analyte inside the pores. So there are two extreme cases in the size exclusion separation. There is a critical size of the analyte above of which the particles are not able to enter the pores of the resin and they are fully excluded from the pores. That means that there will not be effective separation to analytes of this large mass and they will all elute in the exclusion limit or void volume (V_0) of the column. The void volume of a well-packed column is approximate the 30% of the total column volume. Respectively, there is a lower critical mass, in which small analytes will completely penetrate into the pores and thus they will also behave similarly and they will elute at the intra-particle volume or else permeation limit.

In reality interactions between the stationary phase and the analyte lead to a non-ideal SEC mechanism. These interactions are mainly electrostatic interactions between the negatively charged silanol groups and the charged analyte. Hydrogen bonding interactions and hydrophobic interactions between hydrophobic proteins and the resin may occur. So eventually the size exclusion mechanism is driven only from the size of the analyte and the pores of the stationary phase, but the adsorption mechanism is strongly influenced and related to the chemistry both of the analyte and the chromatographic system (ionic strength, pH, stationary phase material) (Kopaciewicz & Regnier, 1982). The conditions can be tuned in order to manipulate the non-ideal interactions taking place in SEC and this will be further proved and discussed in the experimental section as it is one of the

scientific goals of this study, where the use of MS-compatible mobile phases and according to the Hofmeister series of the anions, both chaotropic and kosmotropic will be investigated for native protein analysis.

NON-MASS SPECTROMETRY TECHNOLOGIES FOR PROTEIN STRUCTURAL ANALYSIS.

Nowadays mass spectrometry is introduced as a power tool for protein characterization and for obtaining structural information. However, there are also very well-established technologies for biomolecular structural characterization with capabilities of different magnitudes of resolution. (Steven, 2008) Techniques with low level of resolution provide information about the components and interactions between the macromolecules, whereas higher resolution technologies generate spectra of more detailed structural information. XRD (X-ray diffraction) and NMR (nuclear magnetic resonance) are of the highest level resolution techniques, which can reveal precisely the atomic structure of macromolecules and by extend the protein structure. Despite the fact that XRD gave a huge amount of structural information about the biomolecules, the main question of interest is still unsolved and remains a big challenge, as the biomolecules in order to be analyzed are captured in a solid crystalline environment and not under physiological conditions, which is a far more complicated environment. So with NMR it is claimed that a more 'native analysis' of the proteins is approached, as it allows the determination of atomic structures of biomolecules and smaller complexes in solution, under almost physiological conditions (pH, ionic strength, temperature). Compared with other routine analytical techniques for protein analysis (MS, X-ray diffraction, circular dichroism spectroscopy, Fourier transform IR spectroscopy), NMR has a main disadvantage regarding the method sensitivity, as milligrams of sample are required for significant data collection. Recent developments have improved NMR sensitivity and a sample volume of 6 μL is achieved (Wang G. Z., 2014). Although both NMR and XRD are constantly improving and developing, especially as far as the sensitivity (limit of detection) is concerned, in comparison to other techniques they still require a quite large amount of sample. Additionally, they both require highly purified samples, as they are detection techniques and not for separation. On the other hand, it is equally important that NMR, in contrast to MS or X-ray diffraction, is a non-destructive technique.

So XRD and NMR are considered as high level resolution techniques that can provide information on the atomic level of structures and their interactions. However, this level of structural information is not always necessary or easy to obtain, and for lower resolution structural information such as shape or size of high order molecular assemblies a variety of other techniques can be used, such as Cryo-EM and SAXS. These techniques are able to provide information of the secondary and tertiary structure of proteins in their native-like conformations. Recent improvements for cryo-EM were accomplished by overcoming the challenges in sample handling, instrumentation, image processing, and model building. These latest advances in cryo-EM, provided the opportunity for three-dimensional determination of a variety of heterogeneity, size and shape macromolecular complexes that would not be possible to be analyzed with conventional XRD or NMR methods (Dokland, 2009) (Zhou, 2008). Despite the fact that SAXS requires similar sample treatment with NMR and XRD, it has the advantages that the crystallization step is not required and that in comparison with the other techniques is a fast technique (Mertens, 2010) and changes in the environment (pH, temperature, ionic strength etc.), can be monitored (Lamb, 2008).

The abovementioned technologies considered as the classical structural biology tools. However, spectroscopic methods and optical biophysical techniques are widely used for structural protein analysis. Their main advantages over the classical technologies are their sensitivity and the fact that they are not so laborious, reasons that makes them suitable also for more routine analysis. UV-Vis absorption spectroscopy, CD and fluorescence spectroscopy. CD is maybe the most important as it provides direct clarification of the protein structure. The main contribution of CD to protein analysis is the direct determination whether an expressed, purified protein is folded or if under mutation the conformation or stability of the protein are affected (Greenfield, 2006). Despite the fact that CD has the advantage of analyzing proteins in the range of μg or less in physiological buffers, it does not provide high level information that can be derived from X-ray crystallography and NMR.

NATIVE MASS SPECTROMETRY AS A TOOL IN STRUCTURAL PROTEIN ANALYSIS.

Higher-level resolution technologies, especially XRD and NMR, are power tools for the analysis of high order protein complexes and networks as already mentioned above. However, the limitation regarding the required sample size and purity of the sample are still obstacles, despite the great improvement of these techniques. On the other hand the breakthrough in the MS development has establish it as an important technology in the field of structural biology. MS provides structural information on the dynamics of complicated multiprotein systems and heterogeneous samples over a wide range of conditions, also close to the physiological conditions. Not only the developments, but also the coupling of MS with other techniques like HDX (hydrogen-deuterium exchange) (Guttman, 2016) (Duc, 2015), OFP (oxidative footprinting), CXL (crosslinking), IM separation, have been proved as crucial tandem technologies for structural protein analysis. Explaining the principles of all the different techniques is beyond the scope of this report, so only Ion mobility mass spectrometry will be further explained in a dedicated following section.
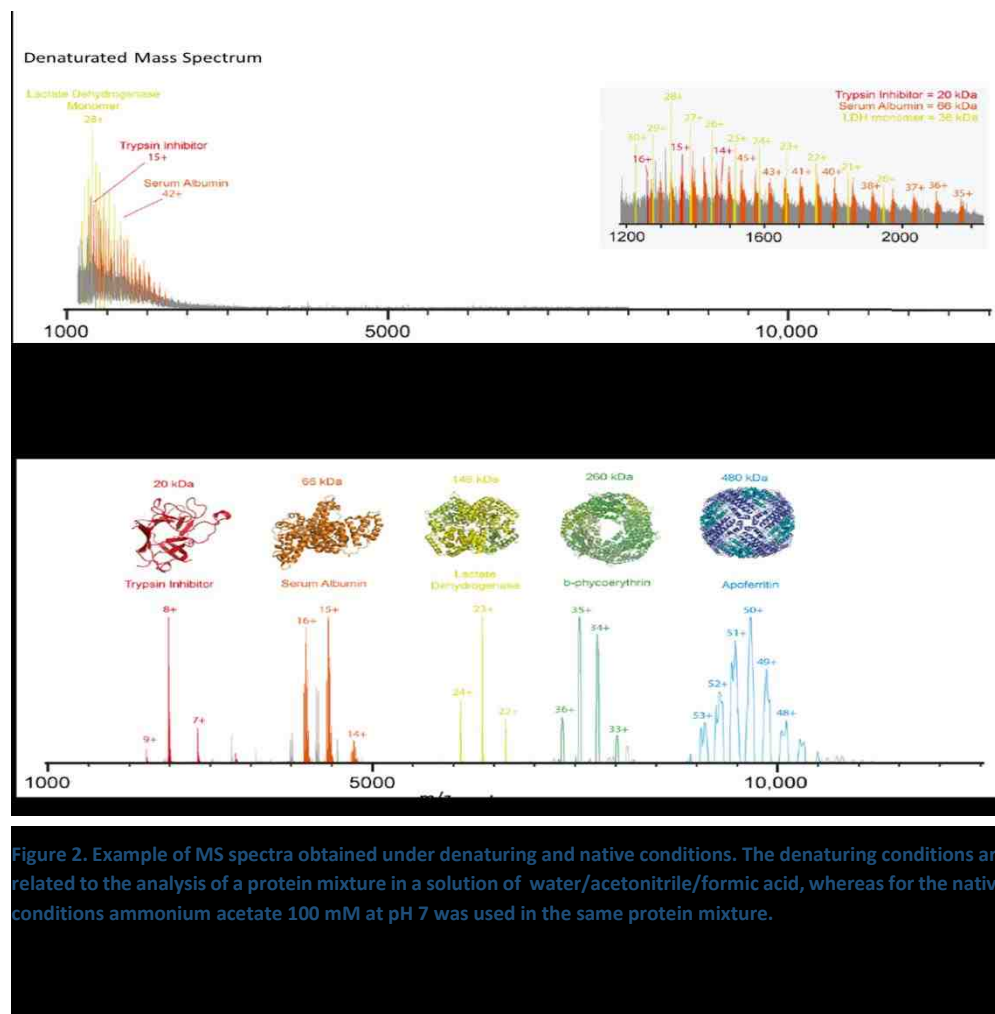
Native MS is based on electrospray ionization, where bio-macromolecules are transferred from a non-denaturing solvent to the gas phase. After a few decades from the introduction of electrospray ionization (ESI), the technological improvement allows this tool to monitor intact or even native proteins. So by the term native Mass Spectrometry is implied that this technique offers the ability to study proteins in their native or at least native-like quaternary structure. The last decade two are the leading groups in the field of native mass spectrometry, the group of A. Heck in the Nederland (Heck, 2008) and the group of C.V Robinson from Oxford (Benesch, Chemical reviews).

The term native mass spectrometry was introduced as a valid term to describe the specific technology, because for many years other terminologies were used such as non-denaturing, macromolecular or supramolecular MS. The problem still remains as different research groups refers to native MS with other terminologies such as non-covalent MS or native spray MS or simply ESI-MS (Leney, 2017). The use of different names to describe the same technology brings a lot of confusion and an explanation of the exact goal and technology behind the term native mass spectrometry is necessary. The term 'native' originally refers to the protein conformation in its natural environment or in other words under physiological conditions, which means inside the cell in specific pH and ionic strength conditions. As a result it is obvious that the actual meaning of native is somehow contradictory with MS, which is a gas-phase method.

The term 'native' for mass spectrometry refers to the conformation of the protein in solution, before the ionization from ESI. So one of the main focuses during native MS is to ensure solution conditions close to the physiological conditions in order to preserve the folded native protein structure in solution. Despite the fact that during MS and especially during detection the analyte will not remain in its native environment it can still provide relevant structural information except just the molecular weight. The fact that structural information remain in the molecule after the desolvation can be proved with ion mobility mass spectrometry (IMS), which will also be further discussed in the following sections.

So in native MS it is important and challenging to be able to mimic as best as possible the biological environment of the proteins. However, having removed the protein from the cells already leads to significant differences and therefore the term 'native' should be carefully used. This stands not only for mass spectrometry but for all the structural biology techniques, which are using it.

Native mass spectrometry became possible only after the development of soft ionization methods, such as electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), which could provide information of high-ordered structure biomolecules. ESI is the most popular ionization technique for bio-macromolecules and its mechanism will be explained in the following sections. Developments such as nanoESI boosted native MS analysis due to the lower sample requirements and higher tolerance in salts. However, in

Figure 2. Example of MS spectra obtained under denaturing and native conditions. The denaturing conditions are related to the analysis of a protein mixture in a solution of water/acetonitrile/formic acid, whereas for the native conditions ammonium acetate 100 mM at pH 7 was used in the same protein mixture.

native analysis is not only the ionization a crucial parameter but also the mass analyzers, as they should cover a broad m/z range, as shown in Figure 2.

The development of triple quadrupole operating in lower radiofrequency increased the m/z range that can be detected. The resolving power of the triple quadrupole in higher m/z values still remains one of the limitations. On the contrary, for time-of-flight (ToF) mass analyzers there is no theoretical higher mass range and both the sensitivity and resolution are efficient. The Q-ToF technology also allows the detection of biomolecules of several million Daltons. The most recent development for improving the resolution and sensitivity is the Orbitrap mass analyzer, which can achieve improved resolution in the m/z range higher than 4000. The emphasis of research related to native mass spectrometry is now focused on scaling the sensitivity and efficiency in order to be able to obtain also topological and stoichiometry information.
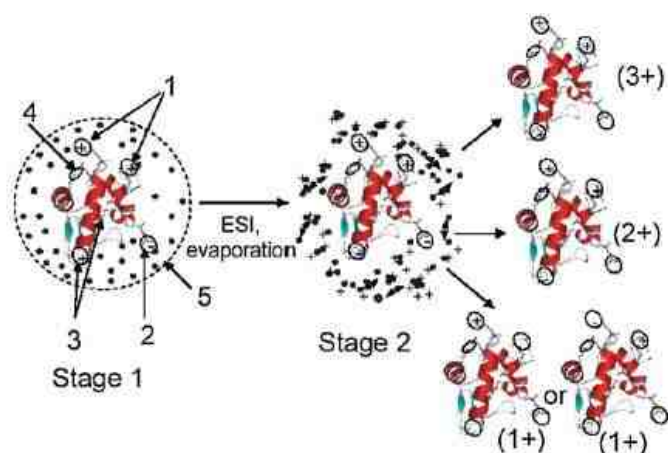
ELECTROSPRAY IONIZATION

It is repeatedly stated that solvent selection is one of the key parameters for native protein analysis and the reason for that lies in the electrospray ionization (EI) mechanism. Initially, analytes in solution are passed through a capillary where high voltage is applied. The droplets of the analyte are generated and as they enter the vacuum interface the solvent is evaporated and the droplets reduce their size due to coulombic fission events. The desolvation process is a key process for the maintenance of the protein structure. It requires an amount of energy that comes from an external thermal energy provided in the system and also energy from the collisions of the droplets. However, it has been proved that for a number of large proteins this process was sufficiently gentle in order to preserve in a high degree their structure (Wani, 2012).

Figure 3. Proposed model for the charge state formation in the ESI-MS as described from (Prakash, 2010).

A proposed model from (Prakash, 2010) regarding the formation of the charge states of a protein during ESI-MS is depicted in Figure 3. The two phases of a protein before and after entering the ESI are described as stages 1 and 2. Stage 1 actually represents the protein and its polar sites in solution. Numbers 1 and 2 show the residues that can actually absorb or release a proton during the desolation, whereas number 3 shows the acidic or basic residues that are part of the folded peptide chain and are not accessible for protonation. Number 4 indicates the salt bridged or ionic interactions between the residues and finally number 5 shows the surface of the solvent. ESI produces multiply charged protein ions and the formation of the different charge states depending on the residues in the accessible area of the protein are depicted with +1, +2, +3 charge states. The residues that will give the charge states of the protein are also related to the negative or positive mode used.

In positive mode the acidic residues are neutralized and the charge states are only due to the basic residues, and in negative mode an opposite way is followed.

As a result this and other extensive studies related to the understanding of the protein conformations obtained from ESI-MS have conclude that the charge-states of the protein depend on its tertiary structure as formed in solution. The fact that under denaturing conditions such as acidic conditions, heat and presence of organic solvent the unfolded protein in solution leads to higher charge states in comparison to the same protein when it is tightly compact in its native state confirmed that mass spectrometry can be used to obtain structural information of the proteins (Konermann, 1998). Despite all the studies around this subject the physical rules govern this phenomenon are still not fully understood. However, it seems that steric changes alter the accessible sites for protonation, basically due to the unfolding of the polypeptide chain, and by extend the pKa values.

Folded proteins reveal a monomodal distribution of charge states, meaning that there is the charge state distributions related to the native/folded conformations are spread in one specific area of the spectrum, whereas unfolded proteins shifts to higher charge states exposing a bimodal-distribution (Šamalikova, 2004), meaning that in the spectrum there are two defined areas related to the folded and unfolded charge state distributions. This was also described from (Cassou C. A., 2014), who investigated the difference in the average and maximum charge states of nine proteins under electrospray ionization with different buffer solutions and concluded the significant increase of both the maximum and averaged charge states compared to their native spectra obtained with lower spray potentials, as shown in Appendix I.

ION MOBILITY MASS SPECTROMETRY

Ion mobility separation coupled to mass spectrometry (IMMS) is a relatively new tool for the analysis of intact protein complexes. IMMS considers as the most recent significant instrumental extension of mass spectrometry after the development of tandem mass spectrometry for native MS. With the coupling of these high resolution techniques several existing limitations at the simple IMS system can be overcome. Initially ion mobility could not be used for identification of unknown compounds and collision cross-section was not sufficient for compound identification. However, the combination of collision cross-section information with molecular mass are more than adequate information for identification. Nowadays, IMMS is becoming more widely used, as it was managed to be proven that the structure of the protein complex is well retained after the transfer from solution into the gas phase (Ruotolo, 2007) (Uetrecht, 2008), which was doubt initially.

The last few years ion mobility mass spectrometry was implemented as structural analysis tool in a variety of analytical fields especially in petrochemical industry, proteomics and metabolomics (Hauschild, 2012), polymer analysis (Hoskins, 2011)as well as environmental and pharmaceutical analysis (Porta, 2013) (Armenta, 2011) (Fernández-Maestre, 2012). So despite the fact that IMS was introduced by Cohen & Karasek at 1970 as an analytical tool, only after its coupling to the mass spectrometer started to be more widely used. The coupling of ion mobility to mass spectrometry adds another dimension in the separation of ions (shape), which allows the determination of molecular weight, fragmentation, clustering and other type of reaction in the drift tube

and makes the two analytical methods complementary to each other. IMMS separation is based on five main processes: sample introduction, ionization of the compounds, ion mobility separation in the drift tube, mass separation and finally ion detection, which will be further discussed in the following section.

The main principle of ion mobility mass spectrometry is that ions are separated based on their m/z as well as their shape and size. Currently there are several types of IMMS instruments commercially available for gas phase separation (Kanu, 2008) (Fernández-Maestre, 2012):

- Drift-time ion mobility spectrometry (DTIMS)
- Aspiration ion mobility spectrometry (AIMS)
- Differential-mobility spectrometry (DMS) or field-asymmetric waveform ion mobility spectrometry (FAIMS)
- Travelling-wave ion mobility spectrometry (TWIMS)
- Trapped ion mobility (TIMS)

Recently Bruker with the introduction of a new IMS analyzer, TIMS-MS (Trapped Ion Mobility Mass Spectrometer), gave the possibility to decouple time as a parameter of the IMS separations, which allows the study of conformationally different trapped molecular ions in the gas phase only as a function of the desolvation time, temperature and bath gas composition (Fernandez-Lima, 2011). The TIMS funnel was introduced into a prototype ESI-TIMS-QTOF (Bruker Daltonics, Billerica, MA, USA) mass spectrometer (Michelmann, 2015). The leading force behind the developments related to IMS is driven for the goal of higher mobility separation and higher ion transmission. The main IM technologies mentioned, differ in order to improve the degree of ion separation before the mass analyzer (Hernandez, 2014). In table 1 a brief overview of the advantages and limitations of IMMS instruments is presented.

Table 1. Advantages and limitations of trap ion mobility mass spectrometry (Fernández-Maestre, 2012)	
Advantages / characteristics	**Disadvantages**
It is operated under atmospheric pressure, which leads to a simple inexpensive instrumentation due to absence of vacuum pumps	Possible contamination by atmospheric vapors
It is considered as an almost universal technique. Effective ionization of variety of samples, organic, inorganic etc.	Spectra are more complex due to different degrees of efficient ionization of different analytes
A wide variety of ionization sources can be used	Limited to analytes with high proton affinity
Gas-phase ion separation	IMMS is not suitable for non-volatile analytes
Adds another dimension in the gas-phase separation, according to the collisional cross section (size and shape)	Size and shape can be influenced from the environment
Because of the speed and sensitivity of the analysis, it is suitable for monitoring in almost real time	Special fast electronical equipment is required
Coupling of IM to MS protects the MS interface, as it limits the amounts that enter the mass spectrometer	Sample size is limited, in order to avoid possible saturation
For better separation the buffer gas/solvent can be alter	Experimental conditions must be carefully reproduced when repeating experiments

TRAPPED ION MOBILITY

As already mentioned above all ion mobility technologies have to undergo the same five main procedures: sample introduction, ionization of the compounds, ion mobility separation in the drift tube, mass separation and finally ion detection.
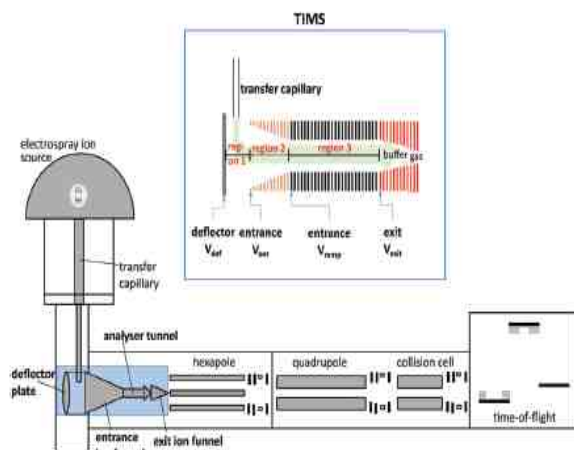
Generally, the sample of interest can be either in the liquid, solid or gaseous phase. However, for ion mobility the sample has to be in the gas phase, which means that sample introduction is basically the conversion of the sample into gas. The selectivity of this procedure is of great importance in order to increase the signal to noise ratio. For that reason, many recent developments combine sample introduction and ionization in one step. Some of the most commonly used ionization sources for IMMS analysis are ESI, desorption electrospray ionization (DESI) and matrix assister laser (MALDI), but also many others can be used (Liu, 2016). The selection of the ionization source is related to the sample matrix and properties. In trapped ion mobility the sample introduction and by extent the sample ionization is being performed with an ESI source. In case the sample is not liquid, sample preparation requires dilution of the sample in aqueous buffers or volatile organic solvents.

The TIMS analyzer consists of three main regions the **entrance funnel**, the **mobility analyzer section** and the **exit funnel** (schematic representation at Figure 4**)**. Right after the generation of the ions from the ESI source, they are transferred at the entrance funnel of the TIMS analyzer through a capillary under the influence of nitrogen gas, which acts as the desolvation gas. For gas-phase ion separation three main steps are required. First of all the ions have to be **collected** and **accumulated**, after the separation takes place and finally the **elution**, before the ions enter the mass analyzer.

It is important that two types of electric fields lead the separation. Initially ions are trapped using radially-confining RF voltages and this is important in order to avoid loss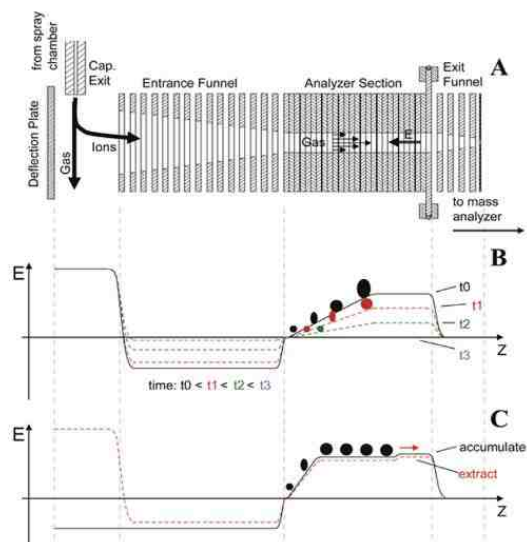 of ions due to vertical diffusion (Ridgeway, 2016). The second important RF voltage is an axial electric field, which accumulates the ions as it counteracts the forces between the field and the drift gas, which later due to decreasing gradient will lead to the elution of the ions. So the main principle of TIMS in comparison to other ion mobility instruments is that the ions are first trapped and accumulated by opposed forces between the moving carrier gas and a gradient electric field step. So ions are accumulated according to their size-to charge ratio in the position where the drift force and the electric field force reach an equilibrium (step 2 accumulation) and because the electric field increases along the analyzer section, ions with different size-to charge ratios will be trapped at different positions, as shown in Figure 5.

Figure 5. Scheme of the three main procedures for ion gas phase separation as described in ref. (Ridgeway, 2016)

The third step is the elution of the ions in order to be further analyzed in the time-of-flight. A decreasing electric field gradient is applied, which leads to the elution of the higher size-to-charge ratios first and the smaller follow.

The most important parameters defining the ion separation in the TIMS analyzer are the drift gas velocity, the ion focusing and the speed of lowering the electric field gradient (electric field ramp speed) (Cumeras, 2015)

After ion mobility separation in (ESI-TIMS-QToF) the ions are further separated and analyzed (Ω/z and m/z) by a Quadrupole (Q) and a Time-of-flight (ToF) mass analyzers, which can be also operated with or without the trapped ion mobility. Because each TIMS analysis takes a relatively short time of a few milliseconds, whereas the time-of-flight spectra are obtained in microseconds, this means that a mobility spectrum reflects the sum of thousands of mass spectra obtained for each ion (Fernandez-Lima, 2011) (Kanu, 2008) (May, 2015).

The final step of the ion gas phase separation is the detection. The same detectors used for regular mass spectrometers are also used in the IMS-MS mode. Detectors record the current change when and ion moves through their surface. Some typical examples are the electron multiplier (eg. photomultiplier PMT), the Faraday cups and plates etc. The results are plotted in a 2D spectrum which contains the information related to the Ω/z and m/z ratios.

FROM SOLUTION TO GAS PHASE FOR SIZE DETERMINATION

The introduction of ion mobility mass spectrometry for native protein analysis was based on its ability to transfer intact species of the protein from solution to gas phase with minimum conformational changes due to the desolvation of the molecules, something that was not possible during ESI (Heck, 2008). Despite the fact that mass spectrometry and ion mobility are powerful tools the transition of the proteins from solution to gas phase is not a simple process. In solution the water molecules play a very important role in the stabilization of the protein conformations. The hydrophobic groups of the protein are directed into the core and they are not accessible by the solvent. It has been suggested that during the transition from solution to gas phase significant conformational changes are imposed and a more "inside out" structure, with the hydrophobic core to be exposed, is achieved (Wyttenbach, 2011). This opinion is based on the fact that vacuum is an apolar hydrophobic medium (Konijnenberg, 2013). On the other hand, according to another theory van der Waals attraction between the polypeptides that remain in the gas phase they are sufficient in order to stabilize the protein subunits. As a result, despite the change from solution to gas phase, molecules tend to preserve their structural information, which means that a possible stress in solution show differences also in the gas phase. The explanation may lie in thermodynamics and the energy barriers for the transition of a structural conformation to a re-forming or breaking of inner non-covalent interactions of the biomolecule, which means that trapped ion mobility conditions are critical in order to prevent structural rearrangements (Liu, 2016).

The last decades there have been significant efforts to theoretically and experimentally prove the differences and alteration imposed in the protein conformation upon desolvation and IMMS has opened the way to this direction (Marcoux, 2013) (Lanucara, 2014). Despite the uncertainty regarding the differences in structure between the in solution and in gas-phase molecules IMMS has been proved to be a sufficiently good indicator that different conformations really exist and that differences in these structures related to their solution environment can be also found in the gas phase (Liu, 2016).

The main principles of TIMS for structural determination are due to the fact that the velocity of the ions is directly proportional to the applied electric field as shown in the Equation 4.

$$K = \frac{V_d}{E} \qquad \qquad \text{Equation 4}$$

Another important equation that describes the mobility coefficient of the analyte ions under low axial electrical field is:

$$K = \frac{3}{16} \frac{q}{N} \left(\frac{1}{m} + \frac{1}{M} \right)^{1/2} \left(\frac{2\pi}{kT} \right)^{1/2} \frac{1}{\Omega_D} \qquad \qquad \text{Equation 5}$$

N is the number density of the applied gas (N_2, He etc.), q, m is the charge and the mass of the ion respectively, M is the mass of the analyte molecule, k is Boltzmann constant and T is temperature in Kelvin. Finally, Ω_D is the collisional cross section of the drift gas averaged.

However, the actual value of collisional cross section after combination of equations which correlates the mobility values and the CCS (Ω) is the following:

$$\Omega = \frac{(18\,\pi)^{1/2}}{16} \frac{z}{(k_B T)^{1/2}} \left[\frac{1}{m_1} + \frac{1}{m_b}\right]^{1/2} \frac{1}{K} \frac{760}{P} \frac{T}{273.15} \frac{1}{N^*}$$
<div align="right">**Equation 6**</div>

z is the charge of the ion, k_B is Boltzmann constant, N^* is the number density and m_1 and m_b are the masses of the molecular ion and bath gas molecule. (Hernandez, 2014)

As we can see from this equation collisional cross section is inversely proportional to mobility (K). However, in trapped ion mobility from Bruker the reduced mobility ($1/K_0$) is used and the reason for that is that K_0 is a normalised value according to standard conditions and $1/K_0$ is directly proposional to CCS, as proved from the following equations.

Reduced mobility is normalised using the pressure at normal conditions p_0 =760 Torr and temperature at normal conditions T_0 = 273.15 K.

$$K_0 = K^* \left(\frac{p}{p_0} \frac{T_0}{T}\right)$$
<div align="right">**Equation 7**</div>

More compact structures with smaller colissional cross section travel faster than ions with more extended conformations because they have less interactions with the buffer gas. However, this is true for the Drift-time ion mobility. In trapped ion mobility the bigger ions are positioned near the end of the anlayser and as a result ion of higher CCS elute faster, thus the reason for reverse mobilograms betweenn DT-IMS and TIMS (Lanucara, 2014).

RECENT EXPLANATIONS OF THE HOFMEISTER SERIES

During this study the effect of mobile phase additives on SEC mechanism and also on the denaturation of proteins will be discussed. An important part of the discussion is the Hofmeister series, which basically orders the salt ions according to their ability to stabilize and solubilize the protein, into kosmotropic and chaotropic ions. Although the Hofmeister effects have been known since the end of the 19th century, they are not fully understood and the explanation of how the salts affect and interact with intact proteins, nucleic acids and other biopolymers in aqueous solutions is still under investigation from the biophysical community. (Record, 2013). For biologists and biochemists the Hofmeister series is quite important as it provides quality information regarding protein precipitation, folding, protein crystallization and protein assembly formation. The importance of understanding the Hofmeister series is even clearer considering the properties that are influenced by ion effects in aqueous solutions, properties such as viscosity, density, refractive index, activity coefficient, osmotic pressure, all of which can influence the native structure of the protein and by extend the results and conclusions of the analytical techniques.

One of the first theory that tried to explain the ionic effects of different salts, regarding the differences in aqueous solubility of proteins and especially the decrease of their solubility when specific inorganic salts were present in solution, namely the salting-out effect is based on the classical Setschenow's equation.
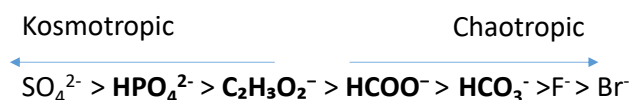
$$\log_{10} \frac{S_0}{S} = \log_{10} \gamma = K_s(\text{salt, solute}) c \qquad \text{Equation 8}$$

From equation above the Setchenow's constant K_s can be calculated from the decimal logarithm of the S_0, which is the solubility of the analyte (protein) in pure water and the S, solubility of the protein under the presence of a salt and c is the salt concentration, and also from γ activity coefficient. The Setchenow's constant (K_s) is positive for kosmotropes, indicating salting out and negative for chaotropes, indicating salting in. However, different cations and anions have different constants and their combination did not align with the observed effects. So despite the fact that this theory governed qualitative information about the high concentration limit of the phenomena, quantification was not possible. After further investigation biophysics conclude that Setschenow's law failed to explain the phenomena in detail, because salt additives in a solution of non-electrolytes is very complex, especially due to the different types of intermolecular interactions between the ions, the solvent and the solute molecules. For instance:

1) The specific composition of the electrolyte (cation and anion pair) plays a significant role on the interactions between the solvent molecules and the analyte.
2) Attractive van der Waals interactions, local interactions between the ion and the analyte mean that the ion is specifically adsorbed. This is also the main improvement of new theories, which take into account also forces that were neglected from the previous ones.
3) The system complexity and the different simultaneous processes, also lead to the reordering of the classical Hofmeister series.

So more recent theories such as the solute partitioning model (SPM) and the interfacial tension concept (ITC) proved that despite the fact that changes in bulk water structure from the salt addition may occur, however they cannot explain the ion specific interactions. So ions being categorized as water structure makers (kosmotropes) or breakers (chaotropes) (Zangi, 2009) was questioned.

<p style="text-align:center">Kosmotropic   Chaotropic</p>

$$SO_4^{2-} > HPO_4^{2-} > C_2H_3O_2^- > HCOO^- > HCO_3^- > F^- > Br^-$$

$$(CH_3CH_2)N^+ > \ldots > NH_4^+ > K^+ > Na^+ > H^+$$

Kosmotropic stabilizing ions	Chaotropic destabilizing ions
Strongly hydrated anions Weakly hydrated cations	Weakly hydrated anions Strongly hydrated cations
⬆ Surface tension	⬇ Surface tension
⬆ Protein stability	⬇ Protein Stability
⬇ Protein solubility	⬆ Protein solubility
⬇ Protein denaturation	⬆ Protein denaturation

Figure 6. Hofmeister series and related properties (Hardy, 2008). The ions presented in bold are discussed during this study.

As a result most theories focused on explaining the direct ion interactions between ions and macromolecules, which resulted in a better understanding on the ability of Hofmeister salts to affect the surface tension, water structure, hydrocarbon solubility and protein stability, yet not fully understood (Zhang, 2006) (Record, 2013) (Lo Nostro, 2012) (Násztor, 2016).

The latest theories have converged in three main possible ways that explain direct anion/cation interactions with the proteins, according to their structure and composition. According to these theorem anions have a higher affinity for amino groups, while cations tend to interact with the carboxylate groups (Násztor, The interfacial tension concept, as revealed by fluctuations, 2016). So except the properties of the protein itself, another explanation lie in the indirect interactions between the ions and the outer water layer of the proteins. According to these theory chaotropic and kosmotropic anions alter the surface tension of the water and by extend the hydrogen-bonding network of the water surrounding the proteins, which lead to a significant change of the hydration entropy and stability of the proteins.

Although it seems that researchers are approaching more and more a valid explanation for the Hofmeister series, there are of course exceptions especially related to cellular processes where a reverse mode of the series is observed. So in a nutshell, although local-protein interactions seem to be the driven mechanism and explanation the extent of their effect seems also significantly dependent on the protein (Han, 2013).

In order to gain a closer insight on the significance of water structure and interactions more recent approaches demand the use of mass spectrometry, where changes in the protein structure can be monitored and correlated to the solution (Kebarle, 2009).

MATERIALS AND METHODS

CHEMICALS

For the calibration of the column a mixture of five proteins was prepared with protein standards from Sigma Aldrich (Schnelldorf, Germany). Thyroglobulin from bovine thyroid ≥ 90% (MW=670 kDa), myoglobin from equine heart ≥ 90% (MW=17 kDa), albumin from bovine serum ≥ 96% (MW= 66 kDa) and uracil ≥ 99% (MW=112 Da) were used.

Other protein samples used in this research study are: the gel filtration standard (# 1511901) from Bio-Rad Laboratories containing thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B12. L-asparaginase was (Paronal ®) purchased from (Nycomed Belgium-Brussels), ubiquitin from bovine erythrocytes ≥ 98% (MW=8.5 kDa) and lysozyme from chicken egg white ≥ 95% (MW=14.3 kDa) from Sigma Aldrich (Schnelldorf, Germany).

The SEC-triple detection calibration was based on the PolyCAL™Standards. So two of these standards were used in this case: the narrow standard was PolyCAL™PEO-24k with Wt=27.60 mg and Mw= 23.850 , Mn= 23.533, IV=0.404 estimated at 30°C in 0.05 M Na_2NO_4. The broad standard was PolyCAL™Dextran with Wt=25.96 mg and Mw=73.227, Mn=56.115, IV=0.257 estimated at 30°C in 0.05 M Na_2NO_4.

For the non-volatile mobile phase used in SEC, sodium phosphate dibasic Na_2HPO_4 ≥ 98.5% (MW=141.96 g/mol) and sodium phosphate monobasic NaH_2PO_4 ≥ 99% (MW=119.98 g/mol), sodium sulphate Na_2SO_4 (Mw 142.04 g/mol) and sodium azide NaN_3 ≥ 99.9%   (MW=65.01 g/mol) were used. All the before mentioned chemicals were purchased from Sigma Aldrich (Schnelldorf, Germany). For the preparation of the volatile buffers used for SEC-MS and direct infusion experiments, ammonium acetate $NH_4CH_3CO_2$ ≥ 98% (MW=77.08 g/mol), ammonium formate NH_4HCO_2 ≥ 98% (MW=63.06 g/mol), ammonium bicarbonate NH_4HCO_3 BioUltra ≥ 99.5% and triethyl ammonium bicarbonate buffer (1.0 M, pH 8.5±0.1) purchased also from Sigma Aldrich were used. The pH was adjusted with ammonium hydroxide (28-30% NH_3), acetic acid ≥99%, or formic acid≥ 97% also purchased from Sigma Aldrich, Merck.

Sodium hydroxide (10 M in H_2O) for the stress of L-asparaginase was purchased from Sigma Aldrich, Merck.

INSTRUMENTS, METHODS AND SOFTWARE

SEC-UV-MS FOR PROTEIN ANALYSIS

The chromatographic instrument used for all the SEC-UV-MS, is the Shimadzu SIL-20AD Prominence Ultra-Fast Liquid Chromatography (UFLC) ('s Hertogenbosch, The Netherlands) system equipped with a pump, auto sampler, column oven and UV detector set at 280 nm. For the analysis of myoglobin, the protein mixture and L-asparaginase, an injection volume of 20 µl and a flowrate of 0.8 mL/min was used. Initially the data analysis using UV detection was performed with LabSolutions software (Shimazdu, 2011) and after LC MS Solutions software (Shimadzu version 3.50.3.18) was used. The SEC column used was a TOSOH (Griesheim, Germany - TSKgel® G2000SW$_{XL}$ 7,8mm i.d. x 30 cm, 5 µm particle size, 125 Å pore size) , which is a diol silica based column

and the guard column was also from the same material and manufacturer TSKgel® SWXL Type Guard Column (6mm i.d. x 4cm, 7μm particle size, 125 Å pore size). The column oven was set at 25°C for the protein analysis and the auto sampler in most cases was set at 4°C, except for the L-asparaginase experiments where the autosampler was set at 8°C. The SEC-MS data for myoglobin and the protein mix was acquired using a micrOTOF-Q (Bremen, Germany) from Bruker with an ESI source which was operated in positive ion mode for all the experiments. For all the SEC-MS experiments a split 1:50 was used in order 2% of the eluent to enter the mass spectrometer and the other 98% to go in the UV detector. The setting were: source temperature 180°C, capillary voltage 4.5 kV, gas flow 4 L/min. Mass spectra were acquired in a range of 100 to 5000 m/z. Data analysis was performed using Bruker Compass DataAnalysis Version 5.0 (Bruker Daltonik GmbH, 2016).

The proposed mobile phase for the SEC calibration was composed of 0.1 M phosphate buffer at pH 6.7 with sodium phosphate dibasic and sodium phosphate monobasic, 0.1 M sodium sulphate and 0.05% (w/v) sodium azide. Each protein for the calibration was in different concentration thyroglobulin (0.2 mg/mL) bovine serum albumin (BSA 1.0 mg/mL), myoglobin (1.0 mg/mL) and uracil (0.1mg/mL) and they were dissolved also in phosphate buffer. For the preparation of the K_d plots 1 mg/mL myoglobin was analyzed, diluted in the same mobile phase used. For the SEC-UV-MS experiments the protein mixture purchased from Bio-Rad Laboratories was used which contains thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B12. The solution of the proteins was prepared according to the instructions of Bio-Rad. The proteins are lyophilized so the first steps include their rehydration with deionized water and gentle swirling. The concentration of this mixture was calculated according to thyroglobulin to 1 mg/mL.

L-Asparaginase of 1 mg/mL was stressed with 10 mM NaOH and 1% w/v ammonium bicarbonate and analyzed with SEC-MS with the above mentioned method both with volatile (ammonium acetate 200 mM, pH 6.9) and nonvolatile phosphate eluent (100 mM, pH 6.7).

Only exception were the SEC-MS experiments of asparaginase, for which a Maxis HD mass spectrometer ESI-Q-TOF from Bruker Daltonics (Bremen, Germany) was used. For the analysis of the native asparaginase two methods were used, as it is also reported in the respective section. Method 1: source temperature 200°C, capillary voltage 4.5 kV, gas flow 4 L/min, IsCID energy 180 eV, transfer time 290 μs, pre pulse storage 50.0 μs. Method 2: source temperature 180°C, capillary voltage 4.5 kV, gas flow 4 L/min, IsCID 120 eV, transfer time 200 μs and pre pulse storage 20 μs.

SEC-TRIPLE DETECTION EXPERIMENTS

The SEC-Triple detection experiments were performed at DSM Resolve, Geleen. The instrument used was a ViscoTek TDA 301 from Malvern Instruments Ltd (Worcestershire, UK), which incorporates Refractive index (RI), Light Scattering detector (RALS-Right angle light scattering & LALS- Low angle light scattering) and Viscosity detectors. The temperature of the detectors was set at 30°C, same as the column temperature to avoid fluctuations and instability of the signal. The injection volume was 100 uL and the flow rate was set at 1 mL/min for both the polymer standards and the protein samples.

For the triple detection experiments 2 L of mobile phases of ammonium acetate 10 and 200 mM were prepared and filtered in order to remove any possible particles that could possibly disturb the light scattering signal. The used chemicals and myoglobin sample are the same as mentioned above from Sigma Aldrich. The polymers used were meant for universal calibration and light scattering calibration and they were purchased from Malvern and they are part of the PolyCAL™Standards. So two of these standards were used in this case the

narrow standard was PolyCAL™PEO-24k with Mw= 23.850 and Mn= 23.533, IV=0.404 at 30°C in 0.05 M Na_2NO_4 and Wt=27.60 mg. The broad standard was PolyCAL™Dextran of Mw=73.227 and Mn=56.115, IV=0.257 at 30°C in 0.05 M Na_2NO_4 and Wt=25.96 mg. These standards are pre-weighted into a glass vial and by just the addition of the correct amount of solvent in this case we added water (density at 20 °C was estimated 0.9982071 g/mL). For these experiments in order to obtain accurate concentrations both the amount of sample and water added were weighted in an analytical balance. The concentration of PolyCAL™PEO-24k was 2.792 mg/mL, of PolyCAL™Dextran was 2.622 mg/mL and myoglobin was 1.0523 mg/mL.

TRAPPED ION MOBILITY SPECTROMETRY

The Trapped Ion Mobility experiments presented in the thesis were performed at Vrije Univeristy, Amsterdam with the new version of TimsTOF (Bremen, Germany) from Bruker. Trapped ion Mobility Spectrometry (TIMS) is coupled to the High-performance QTOF Mass spectrometer from Bruker equipped with an ESI source. Ions are typically generated using an Electrospray Ion Source (Apollo II design, Bruker Daltonics Inc., MA).

Direct infusion experiments were performed with ubiquitin (~8.5 kDa) and myoglobin (~17 kDa). Due to the different size of these protein completely different approaches related to the methods used will be further discussed in the following sections. The software used for the analysis of the data is again Bruker Compass DataAnalysis Version 5.0 (Bruker Daltonik GmbH, 2016).

For the direct infusion experiments of myoglobin the following method was used: source temperature 180°C, capillary voltage 4.5 kV, nebulizer 0.4 bar, gas flow 4 L/min, isCID energy 0.0 eV, transfer time 90.0 μs, pre pulse storage 10.0 μs. For the drift tube: D1 (CAP-DET) -20 V, D2 (DED-DET) -150 V, D3 (DET-FIN) 70 V, D4 (FIN-T1A) 100.0 V, D5 (T1B-T2) 0.0 V, D6 (T2-T3S) 100.0 V, RF 350.0 $V_{pp,}$ Ramp Start (T4-T3) 233 V, Ramp End (T4-T3) 61 V, accumulation time 514 ms, transfer time 2 ms and ramp time 65 ms.

For ubiquitin experiments for native analysis the following method was used: source temperature 180°C, capillary voltage 4.5 kV, nebulizer 0.4 bar, gas flow 4 L/min, isCID energy 0.0 eV, transfer time 90.0 μs, pre pulse storage 10.0 μs. For the drift tube: D1 (CAP-DET) -20 V, D2 (DED-DET) -150 V, D3 (DET-FIN) 70 V, D4 (FIN-T1A) 100.0 V, D5 (T1B-T2) 0.0 V, D6 (T2-T3S) 15.0 V, RF 350.0 $V_{pp,}$ Ramp Start (T4-T3) 233 V, Ramp End (T4-T3) 61 V, accumulation time 5.0 ms, transfer time 2 ms and ramp time 70 ms. Based on the same method and only varying the accumulation time from 5.0 ms to 35 ms and 70 ms the experiments of the dependence of the accumulation time in ubiquitin denaturation were performed.

For the TIMS direct infusion experiments ubiquitin 1 mg/ml was analyzed with ammonium acetate, formate, bicarbonate at 200 mM pH 7.0 and 1% v/v acetic acid. For myoglobin experiments a stock solution of 3.5 mg/ml was used, from which 250 μL were dissolved in 5 mL of each salt solution in order to obtain 10 μM concentration and 1:2 dilution in order to obtain 100 μM.

RESULTS AND DISCUSSION

SEC CALIBRATION

The chromatogram of SEC is an elution profile of the change in concentration (typically measured with UV absorbance at 280 nm for proteins) as they elute according to their size, or more accurately Stokes radii. The void volume (V_0) of the size exclusion columns is the elution volume of molecules that do not enter the pores of the column material but they pass directly through and for the void volume determination thyroglobulin was used (protein 1 in Figure 7). On the other hand very small molecules that fully enter the pores determine the total column volume (V_t), which in this case was determined with uracil (peak 4 in Figure 7). The elution behaviour of the intermediate molecular weight molecules can be expressed as the elution volume V_e , which can be determined from the chromatogram. The void and the total column volume are used for the K_d determination in the next experiments.

As the separation is based on size the calibration curve was constructed using the known compounds in order to be used for the size estimation of unknown analytes (Kostanski, 2004). The calibration curve is typically a third order polynomial line of the logMw against the retention volume, as presented also in the Appendix II. This equation was used for MW estimations during this research.
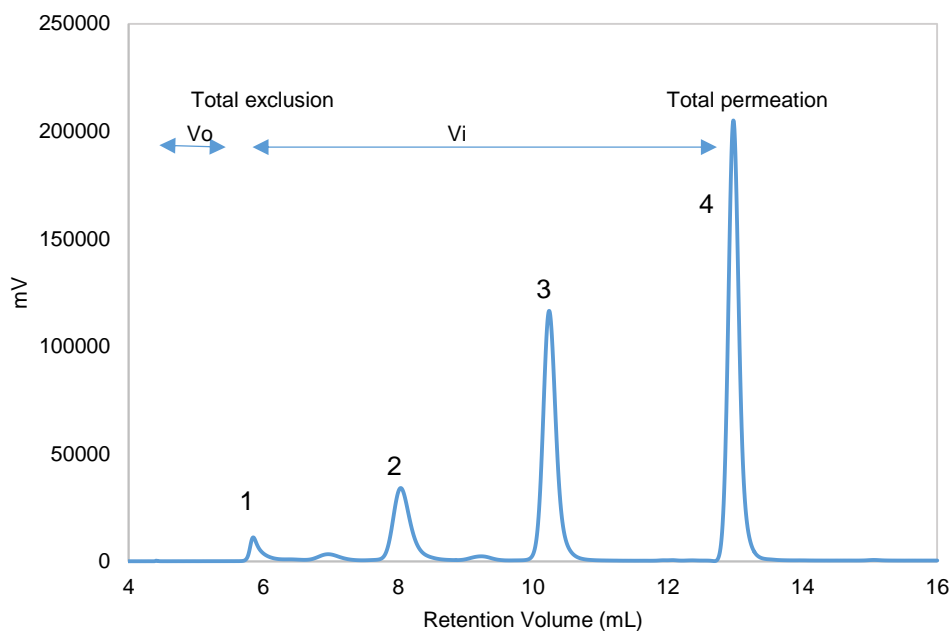
Figure 7. SEC calibration curve of TSKgel G2000W$_X$ with thyroglobulin (0.2mg/mL) bovine serum albumin (BSA) (1.0 mg/mL), myoglobin (1.0 mg/mL) and uracil (0.1mg/mL) and eluent of 0.1 M phosphate buffer at pH 6.7 with sodium phosphate dibasic and sodium phosphate monobasic, 0.1 M sodium sulphate and 0.05% (w/v) sodium azide.

The exclusion and permeation limits are related to the void and total column volume as they also define the size of the proteins or analytes that are not able to be separated. The linear range of this curve, with the intermediate molecular weight proteins (BSA 66 kDa and myoglobin 17 kDa) indicates the appropriate molecular weight range for separation at the specific column, which for our system is from 5 kDa up to 150 kDa. (Hong, 2012)

SEC-UV OF MYOGLOBIN AND MIXTURE OF PROTEINS

In order to investigate the elution behavior of bio-macromolecules in aqueous SEC, myoglobin was analyzed under different conditions of pH (5.9, 6.9, 7.5), ionic strength (10 mM, 25 mM, 50 mM, 100 mM, 200 mM) and both kosmotropic and chaotropic anions (phosphate buffer, ammonium acetate, ammonium formate, ammonium bicarbonate).

The chromatograms of the lower and higher ionic strength buffers are presented in Figure 8, for simplicity purposes. For the lower ionic strength conditions already from the peak shape, where peak broadening and tailing is present the non-ideal SEC interactions can be observed, especially in comparison to the peak shape obtained under higher ionic strength conditions. It is also observed that the type of salt used lead to a different extend of these interactions, as ammonium bicarbonate and formate at low concentrations seem to be unable to hinder the interactions between the analyte and the stationary phase.

The distribution coefficient of myoglobin was calculated and plotted against the salt concentration of the different buffers to visualize its behavior with changes in pH and salt concentration (Figure 8). The K_d factor was used due to the fact that is independent of the column dimensions and as a result it allows the comparison and prediction of the elution behavior of a specific analyte between columns with different dimensions. For the determination of the K_d factor the void volume (V_o) and the intra-particle volume (V_i) were estimated from the most recent calibration curve and the retention volume of the analyte for each particular mobile phase. The K_d was determined according to the abovementioned equation as it was proposed by (Kopaciewicz & Regnier, 1982).

From these plots, where both ionic strength and pH seem to influence the elution behavior of myoglobin the extent of hydrophobic and electrostatic interactions can be evaluated for the different volatile or non-volatile salts. The distribution coefficient K_d equals to 0 means that the analyte is totally excluded from the column leading to earlier elution and K_d higher than 1 indicates absorption on the column leading to later elution (Tayyab, 1991). From the graphs we can conclude that in order to decrease the non-ideal interactions the optimal conditions lie closer to the straight tangent lines of higher ionic strength and pH that is related to the pI of the protein. Despite the fact that the pH seems to play a minor role under high ionic strength conditions, for the intermediate and low ionic strength pH and subsequently also the pI of the protein are important. Myoglobin has two isoelectric points of 6.8 (1) and 7.2 (2). When the pH of the solution is lower that the pI of the protein then the protein is positively charged and ion exchange interactions with the negatively charged silanol groups of the stationary phase take place. Contrary, when the pH of the solution is higher than the pI of

the protein then the protein is negatively charged and this leads to ion exclusion. So with higher salt concentrations the non-ideal SEC interactions can be hindered.

From both the comparison of the chromatograms and the K_d plots it was observed that ammonium acetate performs quite similarly to the phosphate buffer, which is considered as the almost ideal SEC conditions, whereas ammonium formate and bicarbonate seem to deviate. The explanation for this observation lies on the Hofmeister series for the anions, as phosphate and acetate are considered as kosmotropic anions, whereas formate and bicarbonate are more chaotropic anions. As explained also in the introduction this series is not fully understood yet but there is a correlation between these types of salts with protein stability, solubility and the water hydrogen bonding environment surrounding them.
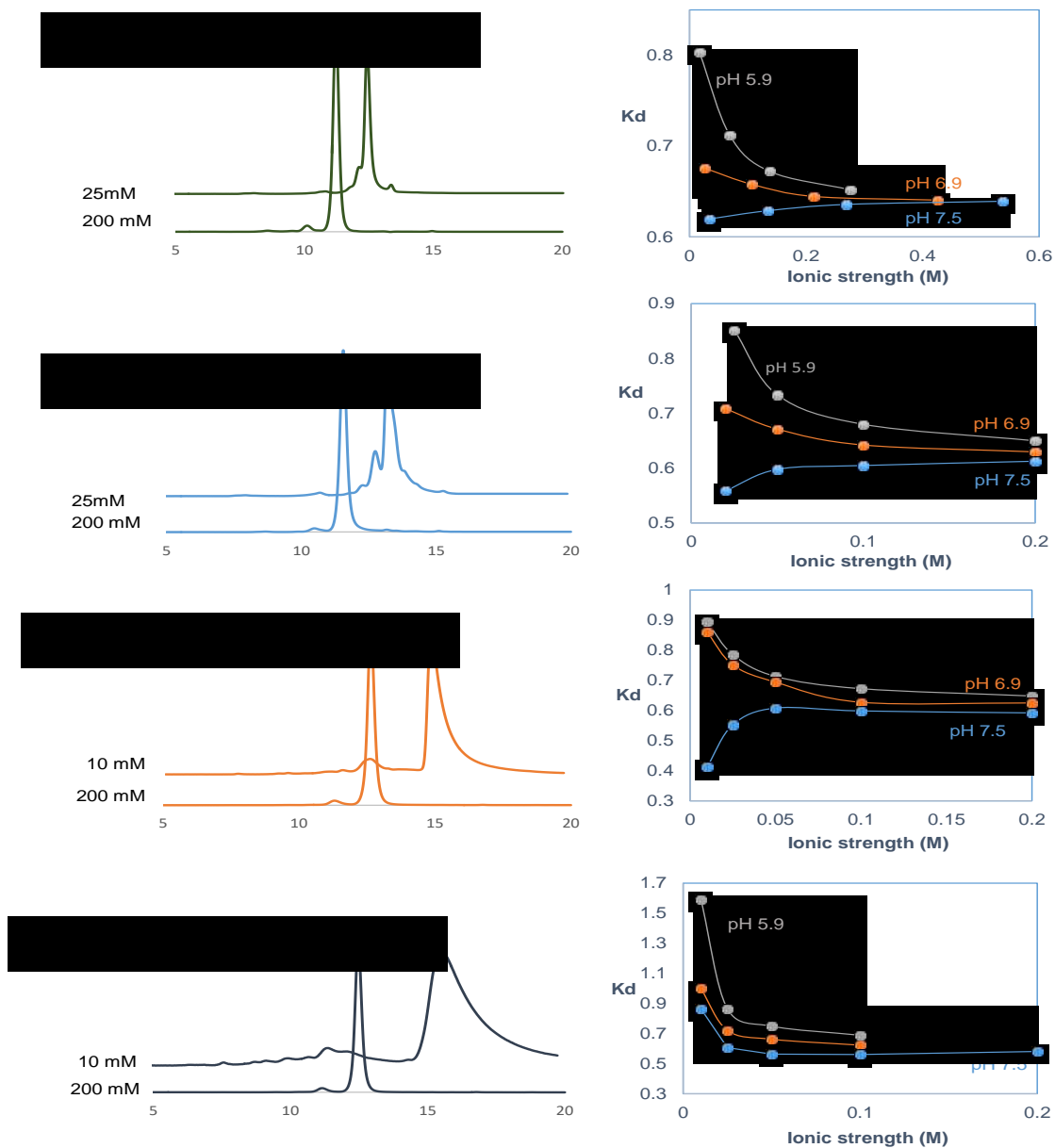
Figure 8. SEC-UV experiments of myoglobin under varying conditions. The chromatograms of higher and lower ionic strength of kosmotropic and chaotropic are presented. K_d plots obtained under the different conditions.

Additionally, another explanation for the deviant behavior of ammonium bicarbonate is that in lower pHs it decomposes to carbon dioxide, (Hedge, 2013, May; Cassou C. A., 2014), which indicates that it is a quite unstable buffer. For the ammonium bicarbonate plot the experiments of 200 mM at pH 6.9 and 5.9 were not performed due to the amount of bubble formation. The pH of ammonium bicarbonate was monitored before and after analysis and a shifting in the pH was observed, whereas the pH of other buffers remained stable also after one or two days. The decomposition of ammonium bicarbonate has an adverse effect on the ionic strength of the solution and by extend to the interactions between the analyte and the stationary phase.

Another observation was due to a shifting in the retention time of myoglobin depending on the type of salt. Initial explanations may lie in the composition of the mobile phase, meaning pH shifting or ionic strength difference, and in order to investigate that further experiments on a protein mixture were performed. In a second stage, trapped ion mobility mass spectrometry was used in order to monitor possible structural differences that might be induced.

Next, a mixture of proteins with different pI's and sizes was tested under different ammonium salts, salt concentrations and pH's. For the investigation of the elution behavior of the protein mixture the extreme conditions of higher and lower salt concentration at the optimal pH for each salt, as revealed from the myoglobin experiments, were chosen. The separation with ionic strength as low as 10 mM is not efficient as can be seen from Figure 9, and as it was also expected. Although, there is an observed trend related to the elution of myoglobin, with higher ionic strength it can be more easily noticeable. At higher ionic strength the elution behavior of the bigger molecular weight proteins is quite similar for the volatile buffers and the phosphate buffer. This can be explained from the exclusion limit of the stationary phase as the bigger molecular weight proteins do not enter the pores and are eluted in the column's void volume. On the other hand, the intermediate molecular weight proteins (γ-globulin, ovalbumin, myoglobin, and vitamin B12) show different elution behavior according to the salt used, which follows almost perfectly the Hofmeister series for the anions, as shown in Figure 9.
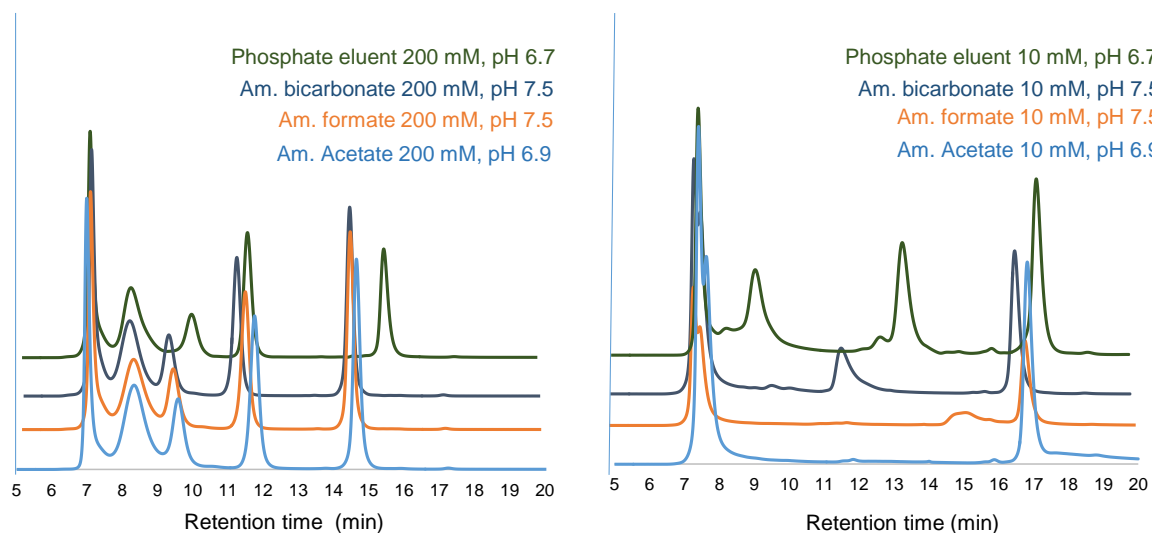
Figure 9. Protein mix chromatograms of different salts (chaotropic & kosmotropic) under "ideal" conditions. On the left, the chromatograms obtained with high ionic strength 200 mM are presented, while on the right the chromatograms obtained with low ionic strength of 10 mM are presented

The explanation for such a behavior might have two additional explanations, except of possible deviations on the composition of the mobile phase. First of all, it is very thoroughly described that the salts used and their properties might lead to structural differences of the proteins. Ovalbumin, myoglobin and vitamin B12 elute closer to the exclusion limit, which means that they penetrate more into the pores, which might be explained from structural alterations. Proteins in the presence of kosmotropic acetate anion keep a higher degree of folding, which means that the hydrodynamic radius has to be smaller and the penetration into the pores is less effective. Contrary, the chaotropic bicarbonate and formate ions lead to protein unfolding and probably bigger hydrodynamic radius and the proteins are more excluded from the pores, resulting in earlier elution.

Another explanation is provided from surface chemistry. Proteins can be adsorbed into the silica surface and there is a number of different parameters that influence the degree of this adsorption. Although, the degree of adsorption on the silica surface cannot be accurately predicted it is proved that not only the primary sequence but also the three dimensional structure of the protein and the isoelectic point play an important role. Because charges can be more easily located in the accessible surface of a protein, these charges and sites are more susceptible to interact with the silica surface charges.

Another very important parameter as has been already indicated also in this research is the aqueous protein environment. The structure and nature of the water around the protein and the silica surfaces can influence the adsorption process (Stutz, 2009). However, the structure of the w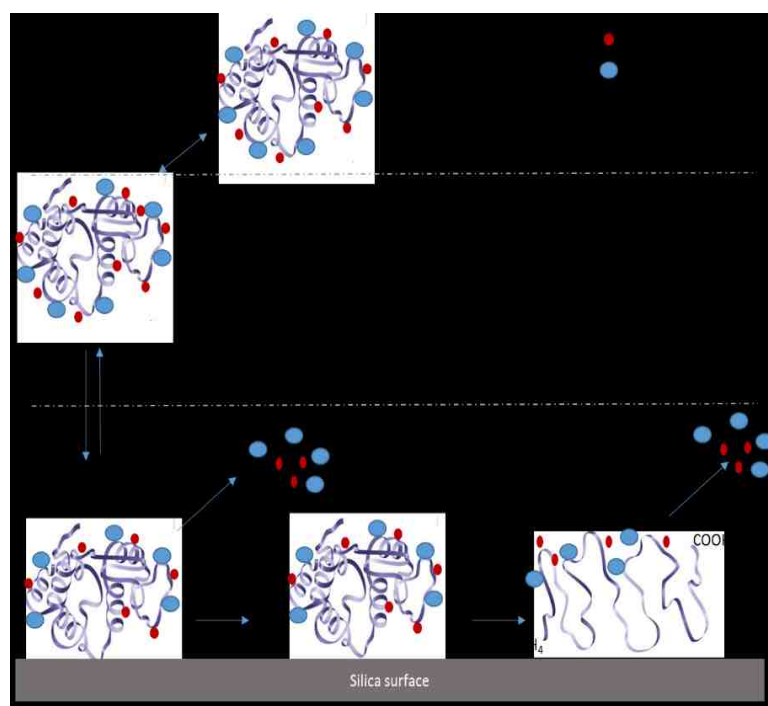ater is a very complex issue of thermodynamics as not only the presence of chaotropic and kosmotropic salts but also properties of the silica surface can influence it, leading to water environment significantly different from bulk water (Vogler, 1998). Another parameter is salt concentration, which can shield the interactions between the protein surface and the silanol groups, and as a result less absorption will lead to less denaturation. The affinity between the protein and the ion is strongly related to the protein and some proteins contain sites that are very eager to ion attachment (Rabiller-Baudry, 2001).

Figure 10. Protein adsorption onto a silica surface as described in the review of (Stutz, 2009) after combining different models describing the protein adsorption in CE silica capillaries

There are many studies and models that focus on explaining the steps that a protein undergo before adhesion to a solid surface (as described in Figure 10). According to these studies protein has to approach the solid surface by diffusion through the different layers of the solvent. So it is assumed that the protein motion is mostly driven by a gradient of the potential between the solution and the silica surface. So after the diffusion of the protein electrostatic interactions between the silica and the sites of the protein that are oppositely charged, but also due to hydrogen bonding, dipole-dipole interactions, van den Waals and hydrophobic effects. Hydrogen bonding interactions occur especially if the silica and the protein are not counter-charged. It is also reported that the extent of conformational changes due to adsorption depends on the inherent stability of the protein but also of the environment, pH and ionic strength and temperature (Nakanishi, 2001). After adsorption due to later diffusion along the silica surface, aggregation of the protein will be caused.

To sum up, this mechanism in combination with the theory explaining the Hofmeister series of the anions and the effect on protein denaturation and stability seems to explain the fact that with lower salt concentration we can observe higher degrees of denaturation also in the SEC-MS, as the protein is less shielded and electrostatic interactions, adsorption and denaturation into the silica surface are prompted to happen. The effect of all of these parameters aimed to be explained with mass spectrometry and the comparison between the analyte's behavior under direct infusion and SEC-MS experiments, which will be discussed in the following sections.

DIRECT INFUSION AT DIFFERENT CONCENTRATIONS AND SALTS

Protein analysis under native-like conditions free of organic solvents allows proteins to preserve non-covalent interactions and retain high degrees of folding (Kükrer, 2010) (Sterling , Batchelor, Wemmer, & Williams, 2010). In order to investigate the ionization behavior of proteins with ESI-TIMS-MS and monitor possible structural differences related to the salt used, direct infusion experiments were performed. In this section only the MS results of these experiments will be discussed. The different buffers of volatile salts were used, ammonium acetate, formate and bicarbonate at different concentrations (10 mM, 25 mM, 50 mM, 100 mM, 200 mM) at pH's close to physiological conditions with myoglobin. For simplicity only the results of the extreme conditions of 10 mM and 200 mM at pH 6.9 for ammonium acetate and 7.5 for ammonium formate and bicarbonate are presented. For these experiments, due to low sensitivity a high accumulation time (513 ms) had to be implemented.

Myoglobin as the model protein for these experiments is a very appropriate protein as the conformational changes native, denatured and refolding conditions can easily be monitored due to the presence of the heme group, which under denaturation is released. Different salts showed different intensities of native (8+, 9 +, 10+) and denatured charge state distribution of myoglobin. Protein folding leads to lower charge states, which conclude to better mass separation and enhanced signal at higher m/z (Zubarev, 2013). Ammonium formate and bicarbonate gave higher charge state distributions than ammonium acetate, meaning that they induced protein unfolding. Bicarbonate showed higher intensities of denatured charge state distribution, than formate, as bicarbonate enhances protein unfolding during the electrospray ionization.

The most abundant peaks for ammonium acetate were the 8+ and 9+, which also according to the literature can be correlated with a more native state of myoglobin. These results are consistent with the general observation that the gaseous ions from a protein at its native conformation in solution always carry lower charge than those from its denatured counterpart (Feng, 1993).
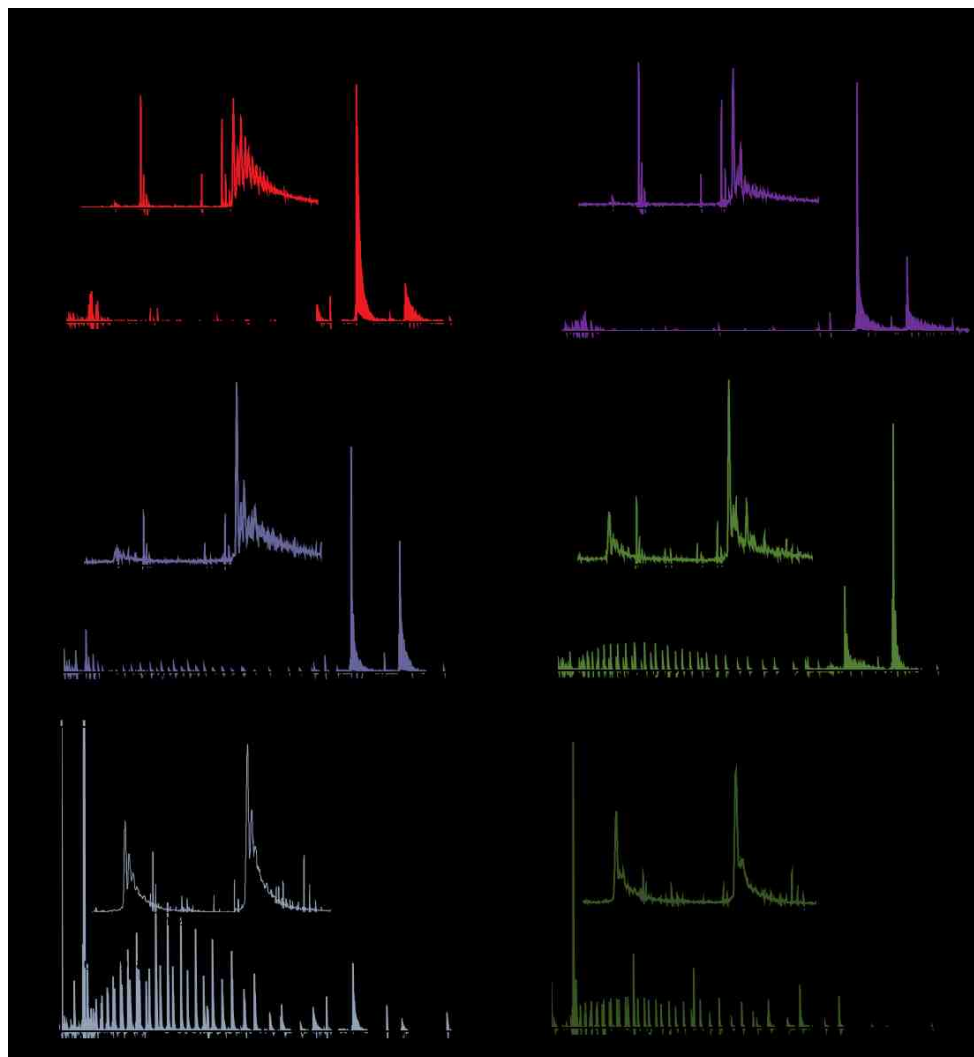

Figure 11. Direct infusion experiments on ESI-TIMS-MS using ammonium buffers of 10 mM and 200 mM at pHs close to the physiological conditions. Holo-myoglobin indicates the presence also of the heme group, whereas apo-myoglobin is without the heme.

The ionization behavior of myoglobin seems again to follow the Hofmeister series of the anions. In parallel also the effect of the salt concentration in the denaturation of the protein and the ion suppression of the signal can be explained from the main principles of the ESI ionization source. Different salts can influence the solution environment and alter the association/dissociation equilibrium of the protein. So there is an optimum concentration range of volatile salt additive, which is also analyte related. (Lifshitz, 2006)

The deviant behavior of ammonium bicarbonate in comparison to ammonium acetate and formate has been again recently reported from (Cassou C. A., 2013) (Hedges, 2013) but the exact mechanism still remains

unclear. One explanation for this behavior is that during the last stages of ESI ionization bicarbonate decomposes into carbon dioxide. Because of the bubble formation bicarbonate leads to supercharged protein species, even in pHs close to the physiological conditions, where proteins are considered folded in a bulk solution. These studies have reported that this effect can be modulated by altering the ESI capillary voltage at lower values and adjusting the source temperature.

The followed trend of the results obtained from ESI-TIMS-MS regarding the effect of salt concentration and nature of salt is consistent with the results obtained from ESI-MS of a previous student working on the same project (Malheiro, 2016), as shown in appendix III.

Although this study focuses mainly on the effect of the chaotropic and kosmotropic anions in both the elution and ionization of proteins, it is also important to understand and investigate the effect of the cation. Both the cation and the anion of a salt influence the protein stability, but it was observed that the anions have a more significant impact that cations as reported in literature (Cassou C. A., 2014). In order to compare the effect of the cations and anions direct infusion experiments were performed in ESI-MS using four proteins (ubiquitin, lysozyme, myoglobin, BSA) of varying molecular weights in solution of ammonium acetate, bicarbonate, formate and triethyl *ammonium* bicarbonate (50 mM, pH=7.0).

Triethyl ammonium bicarbonate was chosen due to the fact that it is being used for purification and preparation purposes. This study was based on the research of (Lemaire, 2001), who also investigated the effect of the cations in the stability of the proteins during ESI using ammonium bicarbonate and triethyl ammonium bicarbonate. As it is reported the difference between ammonium salts and triethyl ammonium salts is the different gas-phase acidity/basicity of thiethylamine (221 kcal/mol) and ammonia (193 kcal/mol), which can interact differently with the basic protein sides. Based on the basicity of ammonia in comparison to the triethyl amine, arginine, lysine, histidine sides can be more effectively protonated, without actually being able to quantify, and thus leading to higher charge states.
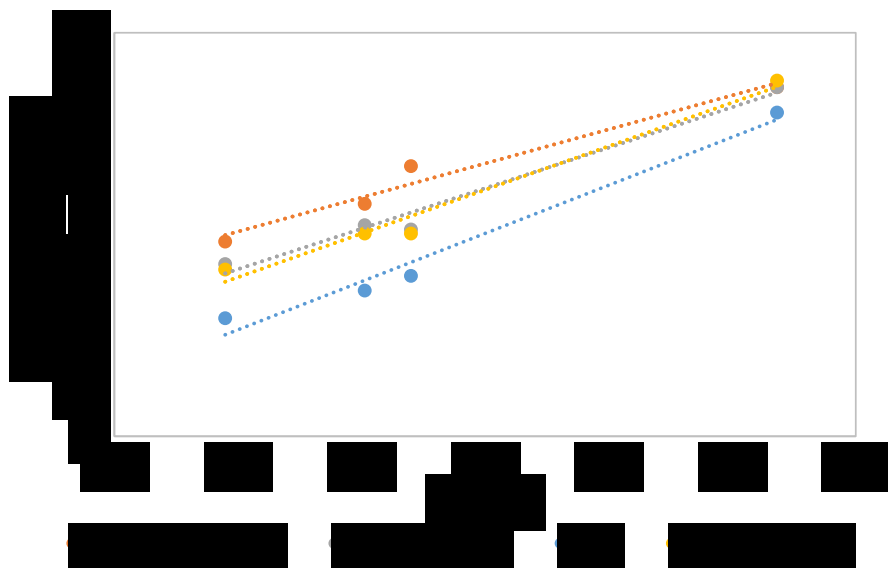

Figure 12. Direct infusion experiments of ubiquitin (8.5 kDa), lysozyme (14.3 kDa), myoglobin (17.5 kDa), BSA (66.5 kDa), under ammonium and triethyl ammonium additives.

The results are consistent with (Lemaire, 2001) not only for ammonium bicarbonate and triethyl ammonium bicarbonate, but the effect of the cation is significantly different in comparison to all the cation counter-ions, as shown in the following graph. This comparison is based on the average charge state of each protein and not only the most abundant charge state as used in Lemaire's study.

TEAB gave for every investigated protein reduced average charge states in comparison to the ions formed by the ammonium salts. Despite the fact that triethyl ammonium bicarbonate is now proved to lead to lower charge states and thus being useful for studying proteins or noncovalent protein complexes, like myoglobin, it was also observed that the sensitivity drops significantly due to the ion pairing effect of trimethylamine, which leads to significant ion suppression. This effect was not further discussed in the previous study of Lemaire, but it might be a very important limiting fact for universal use of triethyl ammonium bicarbonate.

SEC-MS-UV OF BIOMOLECULES

As already reported in the above section related to SEC-UV experiments, during the investigation of the effect of the ionic strength of volatile mobile phases (ammonium acetate, formate, bicarbonate), differences in the elution behaviour of almost all the intermediate molecular weight proteins were observed during SEC. This behaviour was correlated not only to the effect of ionic strength in the interaction mechanism of size exclusion but also the inherent propertied of the eluent seemed to be of high importance (kosmotropic and chaotropic salts) and also the chemistry between the analyte and the silanol groups. The fact that proteins can undergo significant changes according to the solution conditions, meaning denaturation and thus alteration of their native structure, indicated that an extra resolution is necessary in order to identify those changes. As a result size exclusion chromatography was coupled to a micrOToF mass spectrometer in order to gain a better insight on the protein behaviour during analysis.

The conditions for these experiments, presented in Figure 13 represent the almost ideal and non-ideal size exclusion as was investigated prior to this analysis. The main conditions chosen were with 10 and 200 mM under the pHs that were considered to have the best peak shape and separation.
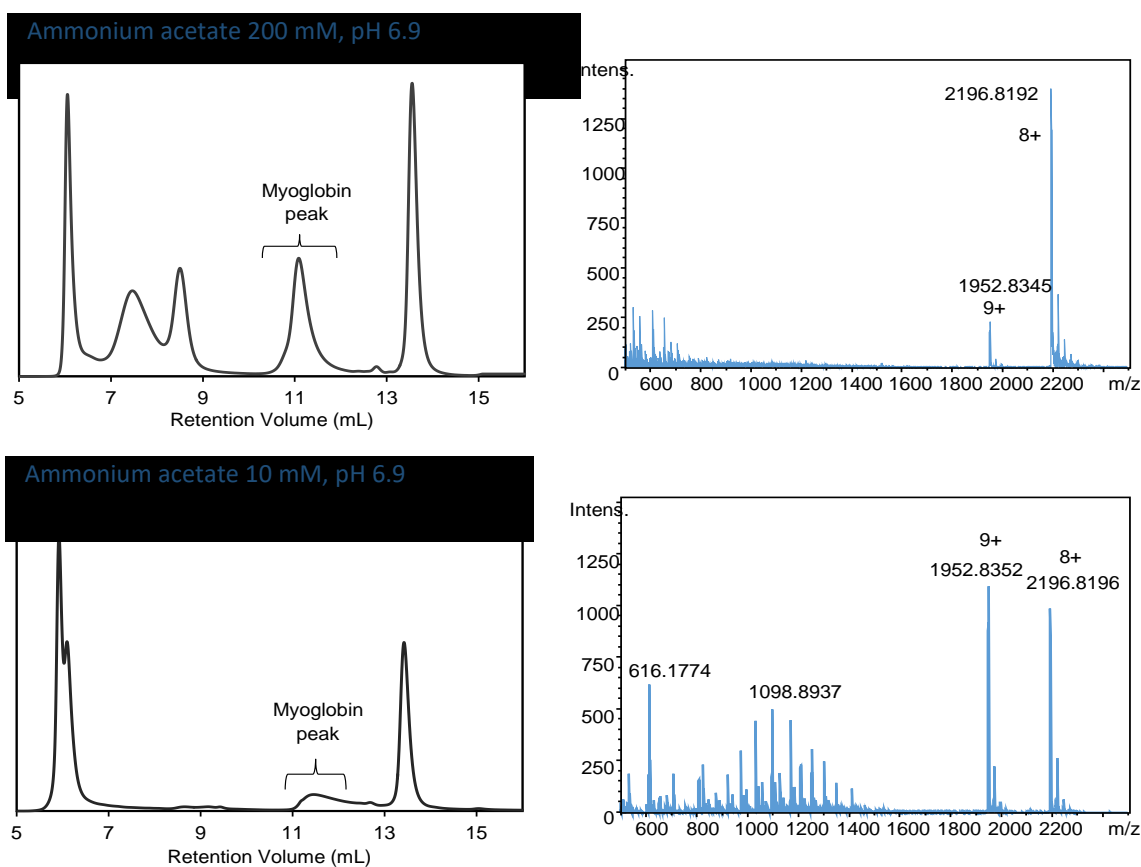

Figure 13. SEC-MS of protein mixture analyzed with ammonium acetate at 200 mM and 10 mM. The spectrum on the right is of the averaged myoglobin peak.

So ammonium acetate 10 and 200 mM at pH 6.9, ammonium formate and bicarbonate 10 and 200 mM at pH 7.5 were investigated (data shown in Appendix IV).

The mixture of proteins was chosen to be investigated under the different conditions however, again the main focus was in myoglobin, since the MS can be tuned either for higher or for lower molecular weight proteins and as a result thyroglobulin, γ-globulin could not be observed, whereas ovalbumin seems to follow almost the same behavior as myoglobin (data not shown here). The ionization of myoglobin, as it was also expected from the direct infusion experiments, follows the Hofmeister series for the anions and more denaturation can be observed with the chaotropic agents, namely ammonium formate and bicarbonate. Ammonium acetate again seems to lead to less denaturation and preserve the native CSD of $[M+8H^+]^{8+}$ and $[M+9H^+]^{9+}$, which correspond to masses of 1952 and 2196 m/z respectively.

However, these results should be further discussed as they are contradictory with the direct infusion experiments. From the previous direct infusion experiments we concluded that higher salt concentration leads to higher degrees of denaturation and also ion suppression. From the SEC-MS experiments the opposite

behavior was observed, as with lower salt concentration the degree of denaturation was higher. In order to approach an explanation about this phenomenon the initial consideration was about the effect of the salt concentration in time. The direct infusion experiments were performed in samples that have been incubated in room temperature, whereas the SEC-MS experiments were performed with fresh samples, produced right before the analysis. So direct infusion experiments were performed for 30 minutes in order to monitor possible changes, data shown in Appendix V. The data showed that incubation time around the same incubation time of the SEC experiments (30 minutes) did not induce more protein denaturation.

Having excluded incubation time as possible cause of extra denaturation, the most possible explanation seems consistent with the theory of the surface chemistry and the present interactions between the protein and the silica surface, in which the salt concentration have a significant impact as it is explained in the SEC-UV of myoglobin and protein mixture section. The shielding of the protein surrounding layer from the counter ions seems to prevent the protein denaturation during analysis. The two effects are contradictory but it seems to co-exist and affect the protein denaturation and unfolding.

SEC-TRIPLE DETECTION

From the contradictory results between the SEC-UV-MS and the Direct Infusion experiments the triple detection was considered as a possible solution that can provide structural information on the in solution protein side and especially the fact that the combined information from LALS, RALS and viscometer, which can lead to the calculation of the radius of gyration (Rg) and hydrodynamic radius (Rh) (Clarke, 2003).
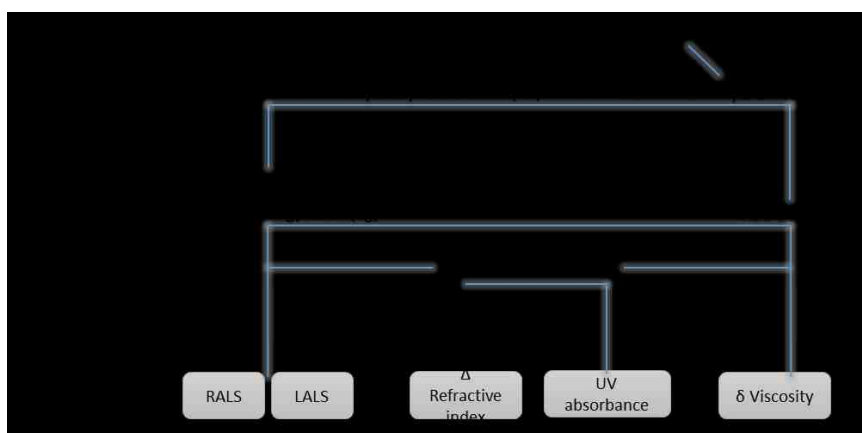

Figure 14. Information derived from direct and indirect calculations based on the triple detection system. Picture obtained from Azo Materials.

Ideally a MALS detector would be more appropriate for the Rg determination, but LALS, RALS and viscometer can also lead to the radii calculation. When Rg and Rh are estimated they can provide a very useful insight on the protein structure, shape, size and protein folding. The goal is to estimate the ratio between Rg/Rh, which provides structural information. For example in case of globular proteins the ration is equal to ~0.775, which basically means that Rg<Rh, but when the protein deviate and becomes non-spherical then Rg>Rh. From the triple detection experiments we intended to calculate the Rg and Rh in order to evaluate differences related to unfolding depending on the kosmotropic or chaotropic salts used. However, due to a number of limitation that will be discussed unfortunately these experiments could not be completed. However, valuable information about the efficiency of the separation of the protein mix tested were acquired.

The first difficulty that had to be tackled was related to the dn/dc 'index increment' of the protein sample and the accurate refractive index of the buffers. The refractive index increment 'dn/dc' is necessary in order to calculate the Rayleigh equation (KC/R_θ), which will provide the accurate molecular weight measurements (Zhao, 2011). The refractive index increment is an important term in the calculation as the scattered light is directly proportional to the square of the dn/dc. As a result in an ideal case for static light scattering experiments the exact value of dn/dc at the exact conditions used should be determined. According to the literature there is a debate regarding the refractive index increment of proteins and whether it has to be considered as a constant or not. In (Zhao, 2011) The use of a good approximation range for the refractive index increment is very useful, because its actual accurate estimation is a rather difficult process that requires also a relatively high amount of pure soluble protein. For proteins analyzed in aqueous buffer the estimated range of

refractive index increment is 0.16-0.20 with an average of 0.185 mL/g (Tumolo, 2004), value which was used for the purpose of this experiment.

Secondly, no accurate weights of the proteins in the mixture were available as it is a commercial premixed calibration standard. So without knowing the exact masses accurate calculations could not be performed. However, for myoglobin and the polymer calibration standards used the accurate masses were known.

For the triple detection calibration both narrow and broad standards are required. So we chose to use PEO standard, which are soluble in aqueous solutions and in order to decrease changes in the dn/dc due to different eluents used we decided to dissolve them in pure water. However, a PEO signal could not be observed when the analysis is performed in 200 mM ammonium acetate. Only a signal in viscosity indicated that indeed the sample was eluting (data shown in Appendix VI). The reasons for this is still to be further explored but the initial explanation is that PEO forms big agglomerates that are retained either from the inline inner filter or they are absorbed in the column and thus the reason that there is no PEO signal in the RI. The fact that there is no re-desolvation of the PEO when using the 200 mM ammonium acetate indicates that PEO agglomerates remain or that this ionic strength is not enough for the elution of the analyte. Unfortunately, the alternative polymer for narrow calibration was pullulan ~110 kDa but this molecular weight is off the limits of the current column used. The results obtained for the broad calibration standard Dextran-T73K are presented in the appendices, and despite the fact that the dextran sample can be detected without the narrow standards no accurate calculations of size or molar masses can be performed.

Since the standards could not be used under the examined conditions, the results could only be used in order to assess the quality of the separation regarding the specific column and conditions. With the 10 mM ammonium acetate buffer the results were consistent with the SEC-UV, indicating that the separation is not efficient and the proteins interact with the stationary phase via absorption, which was already known and expected (results presented in the appendix). It was observed that the proteins elute in the flushing region of the viscometer where interference with the holdup volume for infinite dilution leads to inaccurate conclusions. This can be seen from the negative broad peak at almost 19 mL which interferes with the protein peak at 20 mL, for the chromatograms obtained with 10 mM ammonium acetate. From the analysis of the protein mixture with 200 mM ammonium acetate we can clearly see a better separation and the higher molecular weight
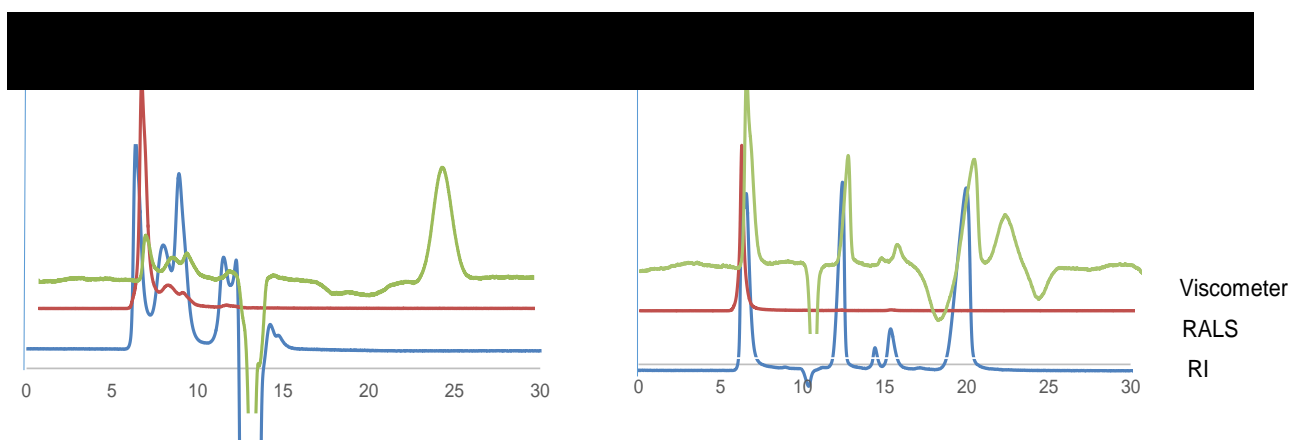

Figure 15. Triple detection of the protein mixture (left) with high ionic strength ammonium acetate and (right) analysis with low ionic strength ammonium acetate.

protein show good RALS signals, whereas between 11-12.5 mL no signals can be observed most likely due to small sizes (Vitamin B12 ~ 1.3 kDa).

Analysis of myoglobin with 200 mM ammonium acetate showed a significantly higher light scattering signal was especially in comparison to the viscosity signal which was smaller indicating a dense structure of myoglobin. Additionally at 6 mL some aggregates could be observed, which show no RI or visco signal maybe due to non-ideal SEC interactions, proving the importance of the triple detection experiments. The fact that myoglobin elutes very close to the permeation limit indicates that the separation should be improved in the lower molar masses range and thus a column of bigger pore size would be more appropriate. To sum up the abovementioned limitations/obstacles should be tackled before having accurate calculations related to the radii of myoglobin depending on the different chaotropic and kosmotropic salts.

SEC-MS OF L-ASPARAGINASE UNDER STRESSED CONDITIONS

The trigger for starting a more fundamental investigation of the mechanism and the conditions of size exclusion chromatography was the unsuccessful try for the analysis of L-asparaginase. In a previous project L-asparaginase under stressing conditions was analyzed with capillary electrophoresis (CE), but in order to verify the outcome of this research complementary information was required. Size exclusion chromatography was used in order to monitor differences in the molecular weight, caused by the basic stressing conditions. However, due to lack of knowledge of the system the experiments could not reveal accurate results. The conditions used for the analysis of the stressed asparaginase were not able to provide a good separation between the lower molecular weight species and the sensitivity of the method was also an issue, as shown in Appendix VIII.

After investigation of the correlation between the mobile phase conditions (ionic strength and pH) and the non-ideal interactions of SEC, the experiment was reproduce but with ultimate goal this time also to identify with MS the lower molecular weight products and chemical modifications induced from the stress. For that reason the separation under volatile and non-volatile buffer conditions were compared. Asparaginase non stressed analyzed under native condition showed, as expected, that asparaginase consists of the tetramer (140 kDa), which is composed of four identical 35 kDa subunits. Upon deconvolution of the mass spectrum of the main peak a mass of approximately 138 kDa was obtained. Two MS methods were used for the analysis of non-stressed asparaginase. However, only one was considered as suitable for native analysis (Figure 16 Method 1) as the other was too harsh and destroyed the tetramer structure (Figure 16 Method 2). The differences between the parameters of the two methods are described in the Materials and Methods section.
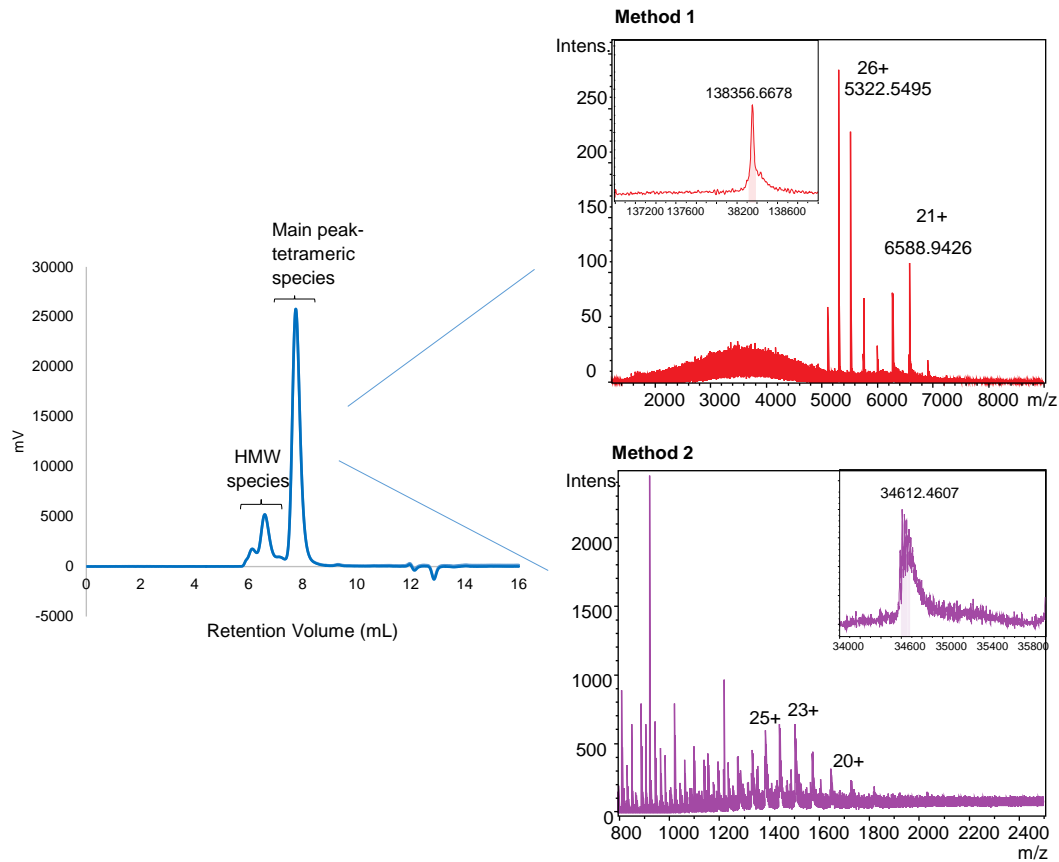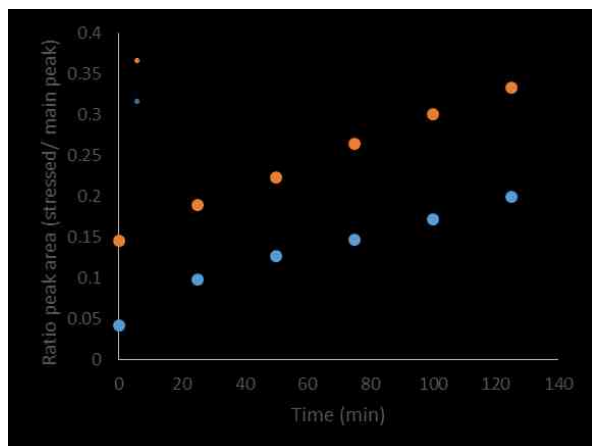
Figure 16. L-asparaginase under natie cocnditions without stress. Two main peaks were observed and can be assigned as the tetrameric species (~ 140 kDa) and the earlier eluting peak as higher molecular weight aggregates. MS method 1 is able to analyze L-asparaginase under native-like conditions, preserving the structure of the tetramer, whereas MS method 2 leads to the collapse of the tetrameric structure into its monomers.

Asparaginase was stressed under two basic conditions, with 10 mM NaOH and 1% w/v NH_4CO_3, and analyzed with the non-volatile phosphate buffer and ammonium acetate. The protein was stressed and analyzed in time in order to monitor possible changes and increase of lower molecular weight species. It was already know that the stress with NaOH is a rapid reaction, whereas with NH_4CO_3 is quite slow. The results of SEC-UV during time for both reactions are presented in the Appendix XI. Upon stress with NaOH difference in the peak area ratio between the main peak and the stressed peak during time was observed depending on the salt used, as can be seen from the graph 1. It seems that the phosphate buffer prevent up to a certain degree the denaturation of the protein, whereas under ammonium acetate a higher amount of the denaturation was induced. The reason for this behavior might also lie on the

Graph 1. Comparison of the ratio of the peak areas between the stressed (lower Mw) peak and the main peak (tetrameric species) for volatile and non-volatile buffer components upon stress with 10 mM NaOH.

properties of the salts, as phosphate considers more kosmotropic that ammonium acetate and thus preserve the protein structure.

From CE analysis three peaks were detected upon stress with 10 mM NaOH (Appendix IX) and in order to verify the assumption that they can be assigned as the tetramer, monomer and deamidated variants of the monomers, SEC-MS was performed. The developed CE method was not MS compatible. For the coupling of SEC with MS high ionic strength (200 mM) ammonium acetate was used as mobile phase at pH 6.9. The mass obtained from the deconvoluted mass spectrum of the main peak showed that it is indeed the tetramer and the smaller peak that increases during time upon stress is the monomer with a deconvolution mass of approximately 35 kDa. Deamidation was not observed. The mass spectra and the assigned peaks can be seen from Figure 17.

Additionally, upon stress with 1% NH_4HCO_3 also more negatively charged, possible deamidated species, were detected, as shown in Appendix X. For the same analysis upon stress with ammonium bicarbonate only the tetramer was revealed as the conditions were not harsh enough in order to decompose it into the monomers and deamidation was again not identified. The high molecular weight species that can be seen from the SEC-UV experiments, could not be identified with SEC-MS due to their low abundance.
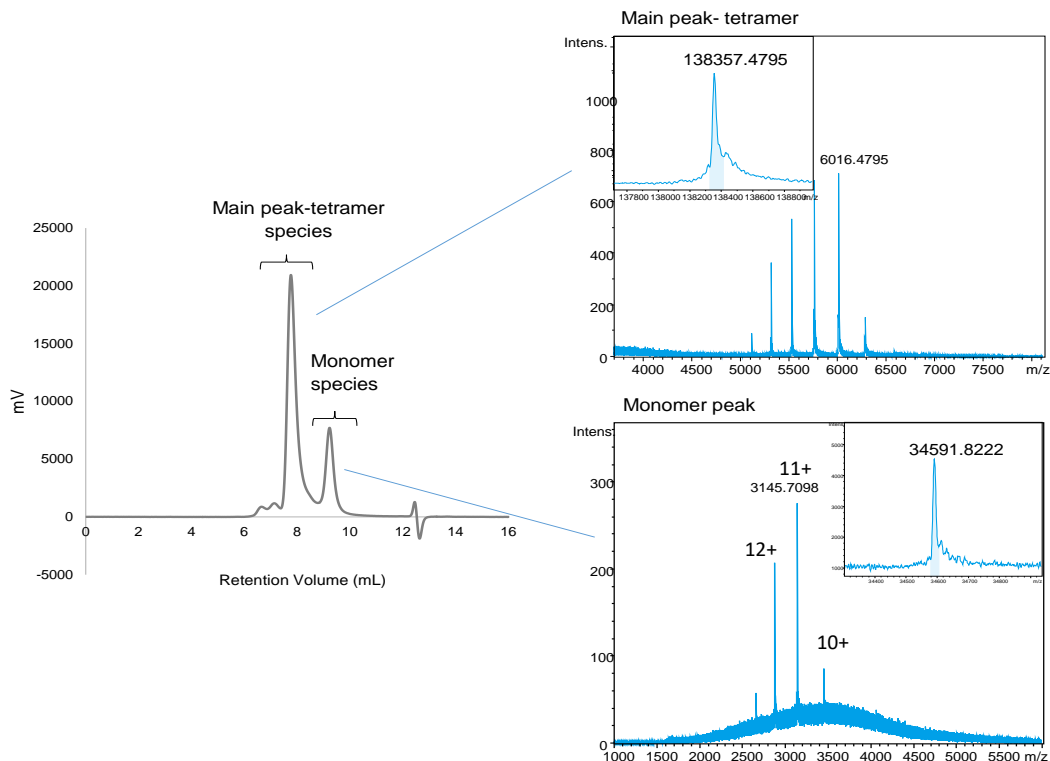
Figure 17. SEC-MS of L-asparaginase upon stress with NaOH and analyzed with 200 mM ammonium acetate, pH 6.9. The two peaks were assigned as the monomeric species of Mw 35 kDa and the tetrameric species of 140 kDa.

TRAPPED ION MOBILITY OF MYOGLOBIN

In this part the results of direct infusion experiments of ubiquitin and myoglobin in TIMS will be discussed, as during the process of trying to find a good method for the analysis of myoglobin a few obstacles were encountered. Having as final goal to perform SEC-TIMS-MS, the conditions had to be optimized in order to be able to analyze myoglobin under the conditions used for SEC, which ultimately means that the final concentration that would be infused in the MS will not be higher than 5-10 µM. The initial experiments with ESI-TIMS-MS were performed with 10 µM myoglobin in ammonium formate 200mM, pH 7.5 and it was observed that myoglobin could not be detected. Because of the low sensitivity obtained when using the IMEX survey mode, which analyzes ions along the full mass range and allows generation of an overview spectrum that is finally resolved by ion mobility, different approaches were investigated. However, for the purpose of this report only the effect of accumulation time and D6 voltage will be further discussed.

Due to the low sensitivity obtained with 10µM myoglobin, a higher concentration of 100 µM was used. The sample was analyzed with and without TIMS (results presented in Figure 18.) in order to understand if the settings used for the trapped ion mobility tube had to be further investigated or it was an instrumentation issue. From the results presented here from a comparison of the intensity of 9+, it is clear that when operating the system with the drift tube there is a loss of sensitivity of approximately 10 times as 9+ without the drift tube separation is approximately 5000 counts, whereas with activated TIMS the intensity was around 750 counts. This might be due to the fact that after ionization ions are transferred and they have to be effectively trapped from the deflector plate in order to enter the drift tube or the other possible explanation if the ions are not effectively enter the qToF.
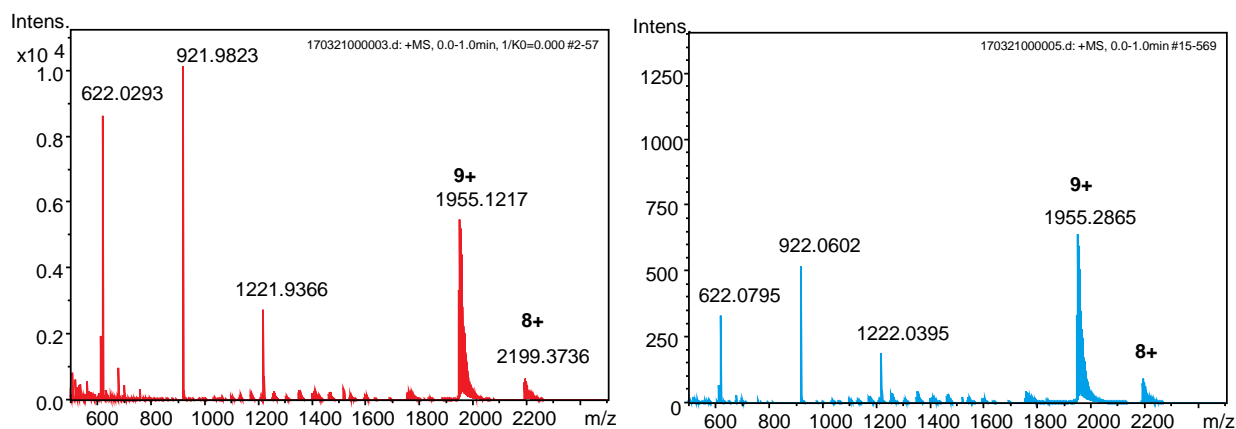

Figure 18. (left) ESI-MS spectrum with intensity of [M+9H]⁹⁺ is approximately 5500 counts and (right) ESI-TIMS-MS with intensity of [M+9H]⁹⁺ approximately 600 counts. Both spectra were acquited from the same sample of 100 µM myoglobin in 200 mM ammonium formate pH=7.5.

The accumulation time of the ions was set from the survey method at 70 ms and the D6 (T2-T3S) was set at 100 V. So at this point different accumulation times were investigated in order to increase the sensitivity. Accumulation times of 50, 100, 300, 513 ms were investigated. Only the 50 and 513 ms will be shown here because the intermediate accumulation times performed similarly to 513 ms. The mobilogram traces also in this case refer to the 9+ charge state, which was also the most abundant ion. The smear present in both cases makes the separation of conformations impossible and especially in case of 50 ms, as shown in Figure 19. The

difference in the intensity is clear as with 50 ms and a concentration of 100 µM the intensity is still low, whereas with higher accumulation times higher intensities can be achieved as more ions are trapped and subsequently detected.
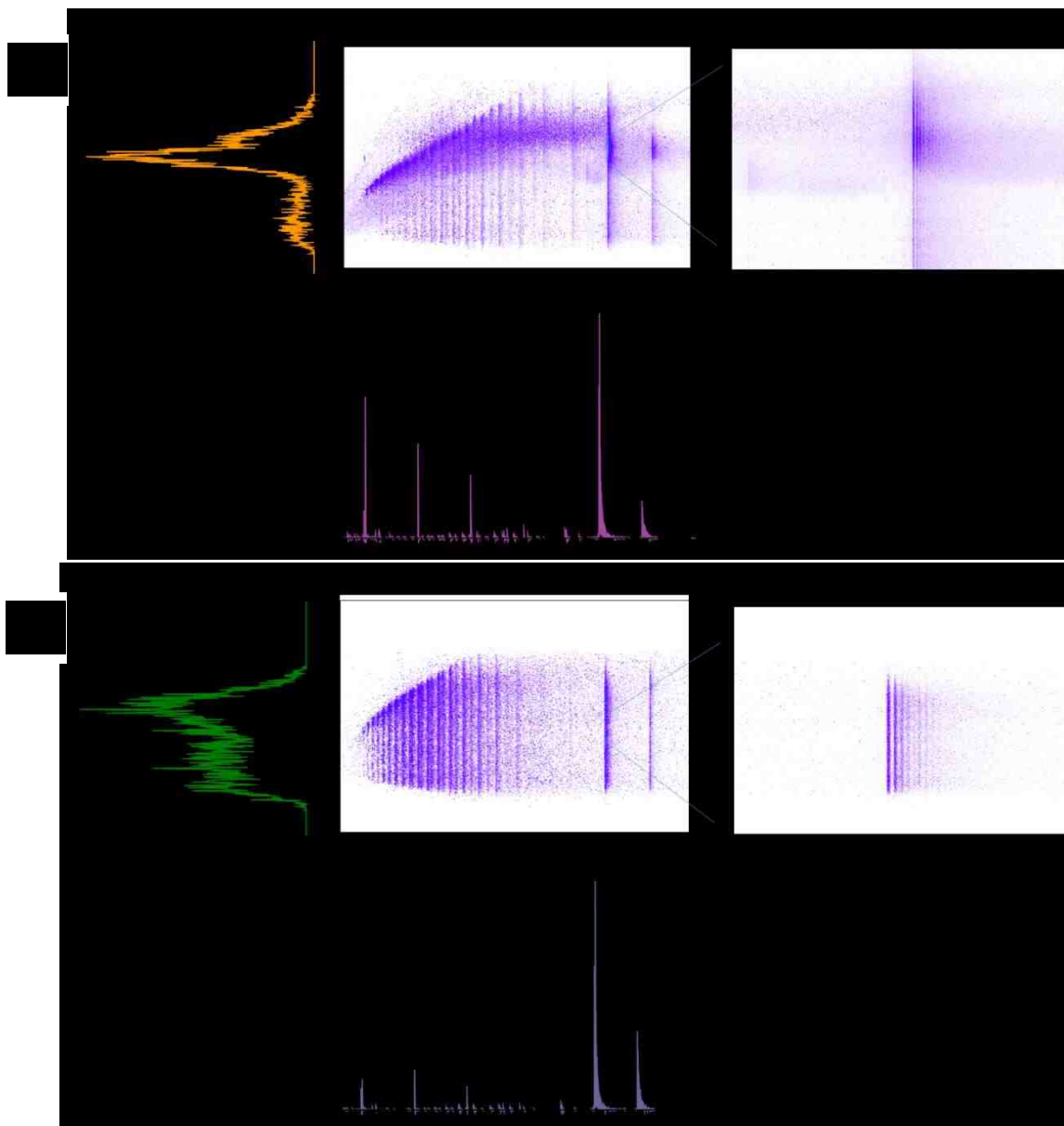

Figure 19. Direct infusion experiments of 100 µM myoglobin in 200 mM ammonium acetate a. shows the heatmap, mobilogram and MS spectrum obtained with 513 ms accumulation time and b. with 50 ms accumulation time.

Because these experiments served two goals, as already mentioned above, first of all to find structural differences between the different salts used, but also to develop a MS method suitable for SEC-TIMS-MS experiments the effect of accumulation time of 513 ms was tested also with a concentration of 10 µM. The MS results obtained from this experiment are being discussed in the section **Direct infusion of myoglobin**.

In order to monitor structural differences depending on the concentration and salt used the charge state of 9+ was chosen as it is the only one present in all examined salts and it is related to the native protein conformation of myoglobin. However, as shown in Figure 20 from the extracted ion mobilograms of the [M+9H$^+$] $^{9+}$ there was not a clear indication that salt concentration leads to different structural conformations. The mobility trace is very broad starting from reduced mobility values 1/K$_o$ approximately of 1.10 up to 1.5 V·s/cm^2 which might indicate that more conformations are present but the method was not optimal in order to induce separation. The same stands also for monitoring differences between the salt additives. More detailed differences might be revealed from the CCS calculation, but unfortunately this was not possible with the current software of TIMS. However, such a high accumulation time is quite likely to lead to interactions between the molecules in the gas phase and as a result separation of different conformations for 9+ cannot be achieved regardless the volatile buffer and its concentration.
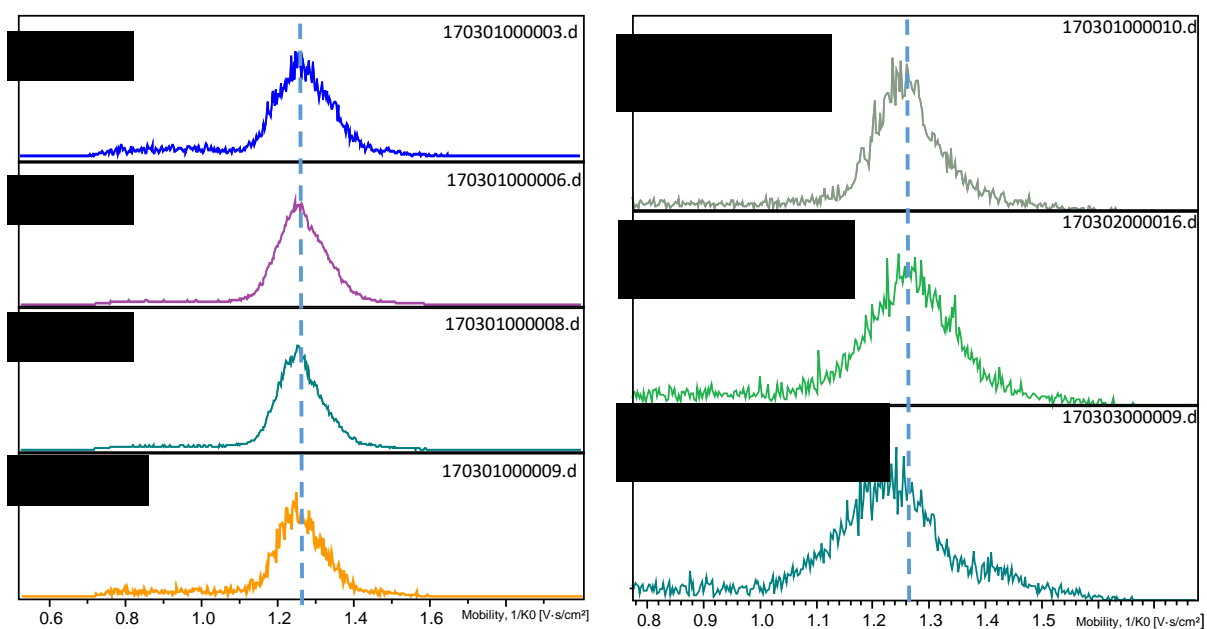
Figure 20. On the left the Extracted Ion Mobilograms of charge state 9+, obtained with ammonium acetate at pH 6.9 at different concentrations from 10 mM up to 200 mM, are listed for comparing the mobility traces and observe possible structural differences related to the ionic strength. On the right the Extracted Ion Mobilograms of charge state 9+, obtained with ammonium acetate, formate and bicarbonate at 200 mM are listed for comparison of mobility traces between the kosmotropic and chaotropic salts

TRAPPED ION MOBILITY OF UBIQUITIN

Because of the struggle to find an optimum method for myoglobin and after advising experts on the field, we came to the conclusion that high molecular weight proteins, because of their size and complexity they perform differently and more knowledge about the settings of the instrument is required. For that reason, in order to gain a better understanding and also to investigate how a result obtained with a previous model of TIMS can be reproduced and even improved with the newer version an experiment with a smaller protein (ubiquitin) was performed. In a previous research of (Liu, 2016) a correlation between structural changes and some critical parameters in TIMS such as the DC potentials at the deflector plate and the entrance funnel and two different RF amplitudes were investigated. They concluded that denaturing of ubiquitin was largely dependent on the amount of translational kinetic energy that an ion will get depending on the applied electric field and the collisions of the ion with the buffer gas particles (see Appendix XIII). They finally developed a method called "soft-TIMS", the structural results of which are consistent with results from elevated-pressure ion mobility drift tubes (Wyttenbach, 2011) and the estimated CCS is comparable to the reported values from NMR experiments related to the native or A-state of ubiquitin (Brutscher, 1997). Having this research as guideline differences in the structure of ubiquitin related to ammonium acetate, formate and bicarbonate were intended to be revealed. The method was set according to the information given in the paper, thus the strength of DC and RF electric fields were adjusted as well as the accumulation and trapping time of the ions. However, since the paper explains in detail the effect of these parameters on the structure of ubiquitin only the results related to the differences between the salts will be reported at this point.

To begin with a few information related to ubiquitin critical for these experiments should be mentioned. Ubiquitin is a protein of around 8.6 kDa and in solution two conformations can exist a more compact and an extended conformation as has been proved from NMR experiments (Brutscher, 1997). The charge states that can be correlated to the native-like ubiquitin are the $[M+6H^+]^{6+}$ and $[M+7H^+]^{7+}$ which correspond to m/z of approximately 1428 and 1224 respectively.

Initially, the most important parameters of trapped ion mobility were determined in order to be able to reproduce Liu's experiment and preserve ubiquitin in an almost native state. Since a newer version of TIMS was used for the current research the method could not be directly transferred and as a result not all the parameters could be similar to the previous research. The method is in detail presented in the Section of Materials and Methods. It is worth mentioning that three parameters were evaluated as crucial parameters for these experiments. The nebulizer gas was initially set at 0.4 bar, but due to very low sensitivity it was increased at 4 bars. The flow of the dry gas was also set at 4.0 L/min, as was advised from (Liu, 2016). Increasing the flow of the nitrogen gas the nebulization efficiency was increased, meaning that the solution of the analyte was better nebulized and the droplets were dried. Another important parameter was the voltage of the D6 (T2-T3S), which might be the potential difference across the entrance funnel. The D6 (T2-T3S) was set at 15 V and it was observed that at higher values of voltage the denaturation of ubiquitin was increased and the separation between the peaks was significantly decreased with result to observe only a broader mobility distribution. Another important parameter that was investigated was the accumulation time of the ions in the tunnel. Three different accumulation times, 5, 35 and 70 ms were investigated in order to monitor possible differences. From Figure 21 it can be seen that for ammonium acetate and acetic the different accumulation

times had a significant influence on the mobility of [M+7H$^+$]$^{7+}$. Especially for acetic acid an accumulation of 70 ms leads to a saturated picture with any separation between the conformations. Another important observation is that for the same charge state with ammonium acetate at the same high accumulation time the ratio between native and extended conformation is almost equal, whereas at lower accumulation time the extended conformation is more abundant. After determination of the abovementioned parameters the experiments for ubiquitin were performed with the lower accumulation time of 5 ms. Two examples of the results obtained from direct infusion of ubiquitin in trapped ion mobility under ammonium acetate and under the harsher conditions of 1% v/v acetic acid are presented in order to show the differences that can be observed. TIMS data is quite compact in terms of information and there are different ways of representation and as a result a clarification of how they are evaluated is necessary. For these experiments 5 min of infusion spectra were averaged in a final heatmap, which is a combination of the mass spectrometry and the mobility data, as presented in Figure 22. From the heatmap the different charge states can be observed at specific mobilities and despite the fact that at first glance no obvious separation between the ions is noticed, when zooming in a clear separation of two and three ion conformations is revealed for ubiquitin in ammonium acetate and water/acetic acid respectively (left heatmap of Figure 22).
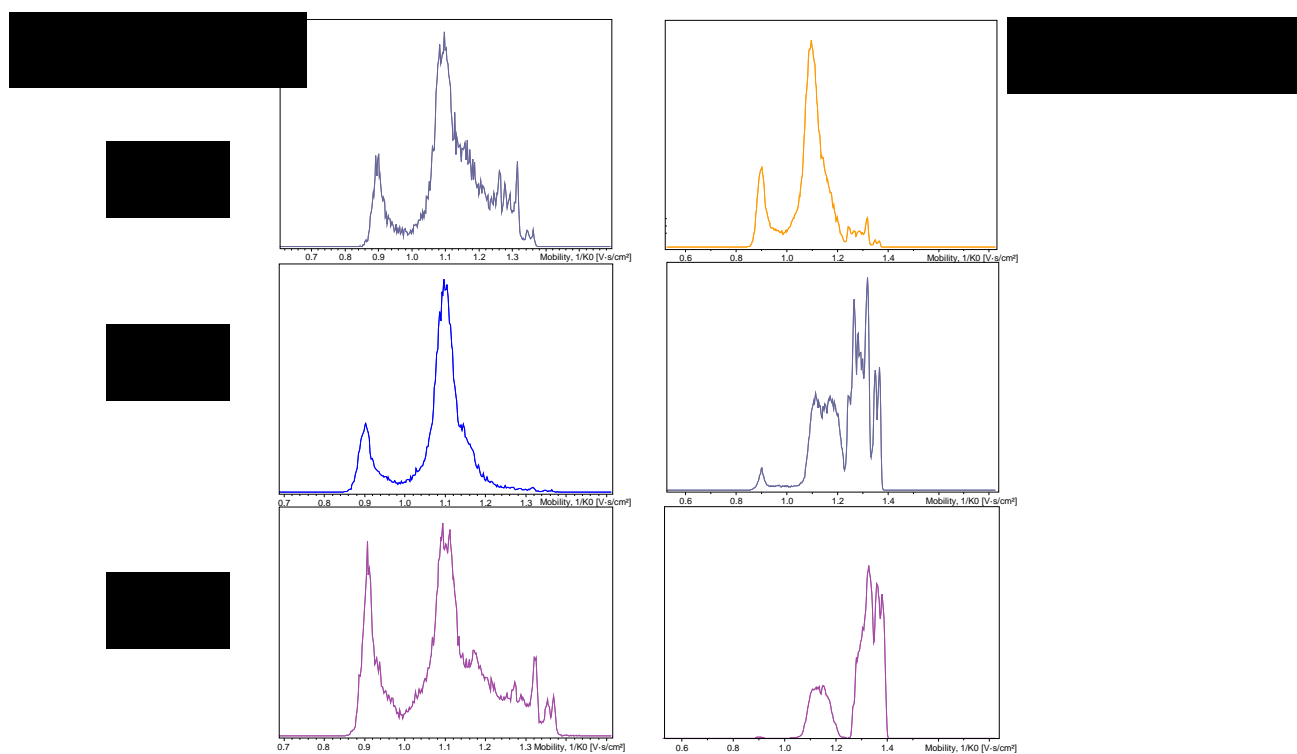
Figure 21. ESI-TIMS-MS of ubiquitin [M+7H$^+$]$^{7+}$ in ammonium acetate solution and 1% acetic acid under varying accumulation time (5, 35, 70 ms) and D6 (T2-T3S) 15 V.
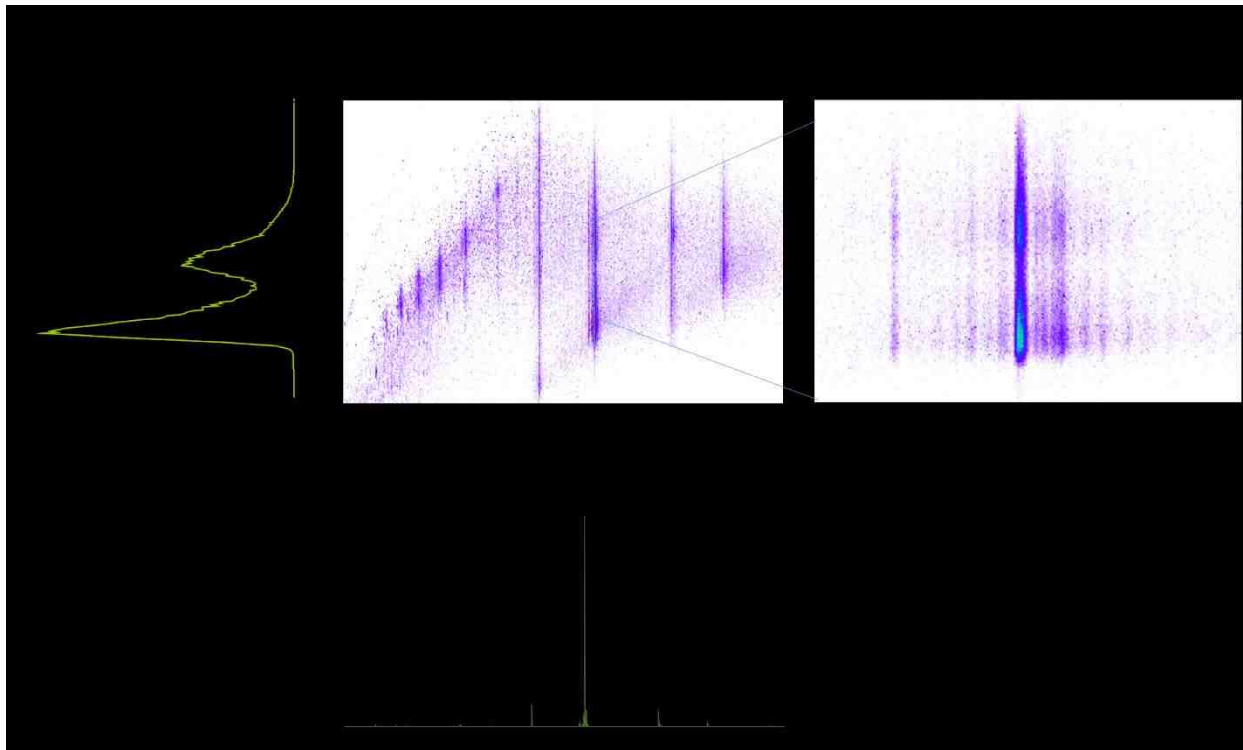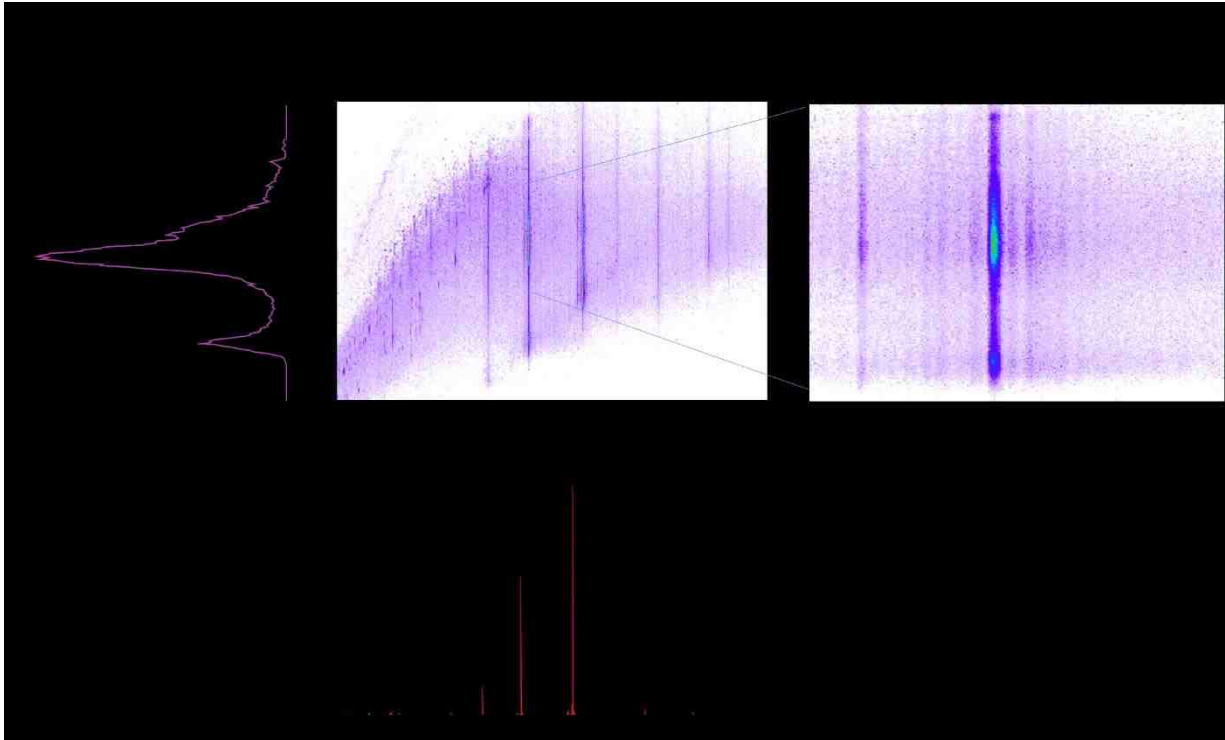
Figure 22. . Examples of TIMS data for ubiquitin under different salt additives. Above picture shows the EIC of 7+ charge state of ubiqutin under 1% acetic acid, where three conformations can be detected. Below picture depicts the EIM of 6+ charge state of ubiqutin under 200 mM ammonium acetate pH 6.9, where two conformations can be detected.
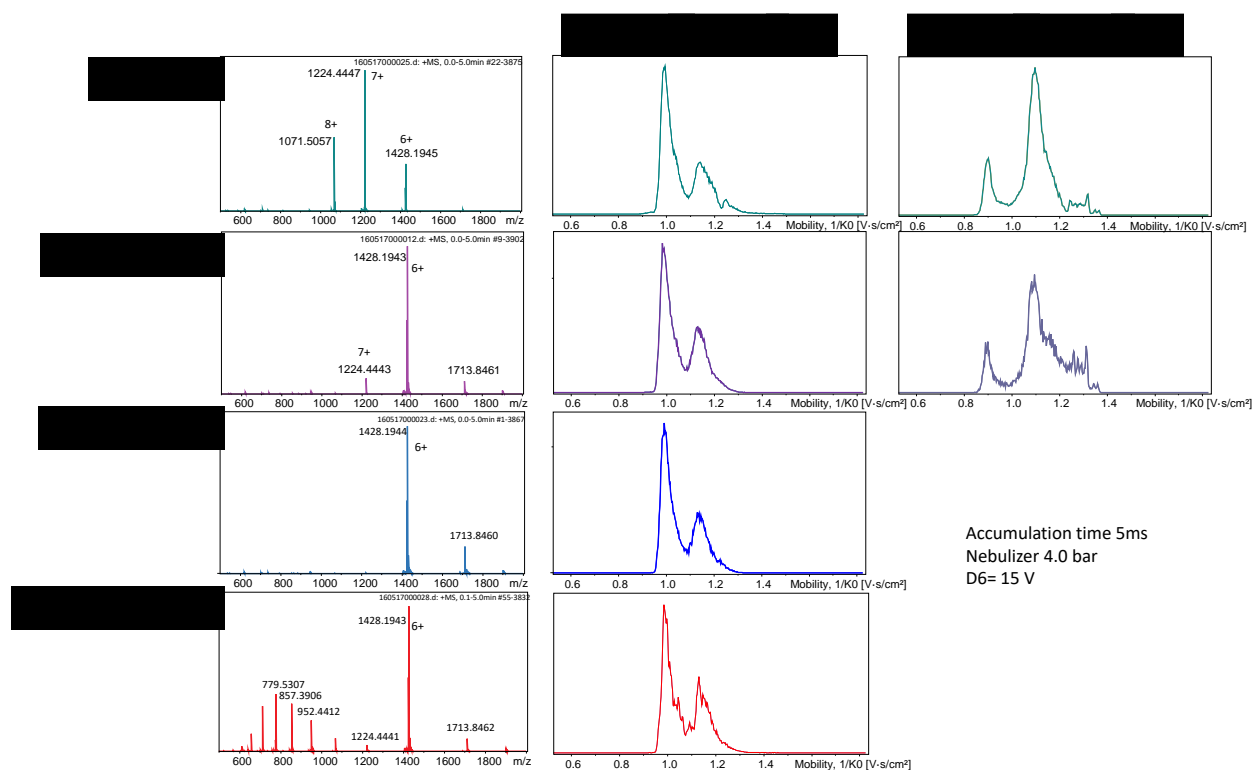
Figure 23. Comparative study for the effect of salt additives (chaotropic and kosmotropic) in the protein conformations. Both 7+ and 6+ charge states are presented depending on their presence in the respective spectrum.

The results obtained are consistent with the observations of (Liu, 2016), meaning that two conformations can be clearly assigned for the 7+ charge state and there might also be a third very low abundant one with mobility of 1.2-1.4 V·s/cm^2, as can be observed from Figures 21 and 22 . However, there is a difference in the ratio between the compact and the more extended conformation as in this case the more extended conformation seems to be more dominant. The reason for that most likely lie in the different parameters used and due to instrumentation variance. Another difference could be observed between the two experiments which is related to the 6+ charge state, for which two conformations could be assigned (Figure 22), whereas Liu reported only one. It was observed that 6+ can be detected in a more compact conformation, which was also the most abundant one, and in a more extended conformation following basically the same trend as 7+ charge state.

Having these information was important in order to evaluate the limits, possibilities and crucial parameters related to ubiquitin and TIMS. So a second goal was to investigate and monitor structural differences related to the chaotropic and kosmotropic salts. For that reason ubiquitin was analyzed in ammonium acetate, formate, bicarbonate and acetic acid with the same TIMS-MS method as mentioned above, and is in detail described in the section of Materials and Methods. The results are summarized in Figure 23.

Unfortunately, the comparison between the different salts could not be based on the 7+ charge state as it was not generated under all the different conditions. Therefore, the comparison was based on the 6+ charge state.

From the data presented above any significant difference between the structures of these ions can be observed and related to the salt used. The only possible difference is that for ubiquitin [M+6H$^+$]$^{6+}$ stressed with 1% acetic acid a very low abundance of a third conformation seems to be present.

However, when the same experiment was performed at higher accumulation time of 70 ms, structural differences could be observed especially for ammonium bicarbonate and acetic acid as presented in Figure 23. The reason for that is, of course, that at higher accumulation time the ions can interact, collide with each other and also with the buffer gas. So, combining also the information obtained from (Liu, 2016), the extend of structural denaturation caused in the trapped ion mobility tube, is related to the DC and RF electric fields, trapping time and accumulation time, which elevates significantly the complexity of this type of analysis and make it clear that is not a straight forward analysis and deep knowledge of both the analyte and the ion mobility are required.
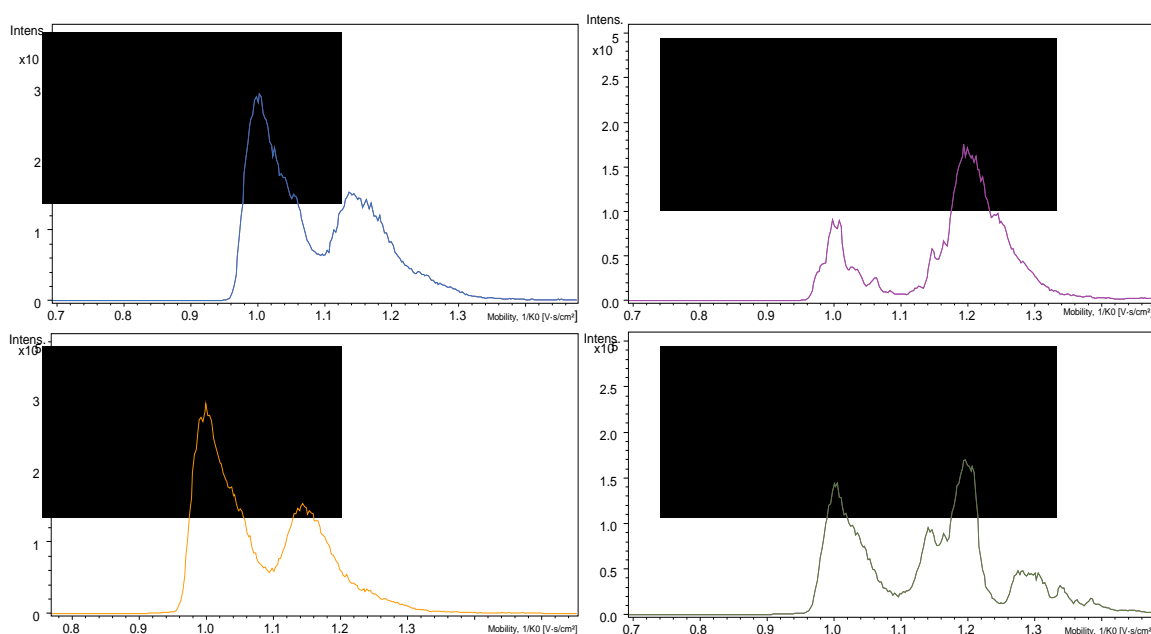
Figure 24. Extracted ion mobilograms of 6+ charge state of ubiquitin under different salt additives and relatively high accumulation time (70 ms). Structural differences can be observed between the different additives, with most likely explanation the interactions of the molecules inside the drift tube.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

From the SEC experiments we can conclude that almost interaction free aqueous SEC can be performed when using high enough ionic strength and despite the fact that under these conditions the pH does not play a major role, it should be adjusted according to the pI of the analyte. At lower salt concentrations the SEC mechanism is mostly driven by the pH of the solution and ion exclusion or ion exchange interactions take place. Both the elution and ionization behavior of bio-macromolecules seem to follow the Hofmeister series of the anions, which indicated that ammonium acetate is a more suitable salt for native protein analysis. From the comparison between the SEC-MS experiments and the direct infusion experiments contradictory results were obtained, as in SEC-MS with high ionic strength less protein denaturation was revealed, regardless the salt used. Additionally, the effect of volatile salts showed different charge state distributions and a different degree of denaturation in mass spectrometry. The difference between SEC-MS and direct infusion experiments was finally explained from the surface chemistry and the possible explanations for the Hofmeister series, that conclude to the alteration of the hydrogen bonding network around the protein and the silica surface. Although both the elution, regarding the retention volume shifting and ionization, regarding the degree of folding, seems to follow the Hofmeister series for the anions in order to attribute the observed differences to structural alterations and not changes in the mobile phase composition (ionic strength, pH, day variance, instrumental variance), statistical validation of the method is required. Additionally, SEC-triple detection or SEC-MALS experiments can also prove structural differences in solution.

Preliminary results of direct infusion experiments of four proteins of different Mw (ubiquitin, lysozyme, myoglobin, BSA) under chaotropic and kosmotropic cations additives, revealed that also the cation effect in the protein denaturation should be further investigated. The use of triethylammonium bicarbonate (TEAB) in ESI-MS lead to very reduced average charge state distributions in comparison with the ions formed in the ammonium bicarbonate, formate, acetate solutions. Despite this nice effect of TEAB, trimethylamine as a known pairing agent seems to cause significant ion suppression, which might be a limiting factor for more universal use. The effect of triethyl ammonium cation on the elution behavior of biomacromolecules should be further investigated.

Trapped ion mobility is definitely not a straight forward analysis and requires very good knowledge both of the instrumentation and the analyte. Three parameters were evaluated as the important parameters for keeping a balance between sensitivity and preservation of the native structure of the protein in the gas phase. The nebulizer gas that can desolvate effectively the molecules and can increase the sensitivity, the D6 (T2-T3S) voltage between the second and the third drift tube base and the accumulation time.

Separation between different structural conformations of specific charge states (6+ and 7+) were achieved for ubiquitin that are also consistent with observations of previous studies. Three conformations were observed for ubiquitin 7+, two more extended conformations and a more compact one. However, neither for myoglobin or ubiquitin structural differences could be observed with trapped ion mobility that are related to the different salts used. Some of these buffer components, especially ammonium bicarbonate lead to denaturation of the protein in the gas phase, which can be also correlated with the high accumulation time in the drift tube. Myoglobin as a higher molecular weight protein was a more difficult analyte that needs to be further investigated after further optimization of the method in order to achieve better resolution and sensitivity that

might lead to the separation between the apo- and the holo- conformations of a specific charge state. Further experiments for optimizing the TIMS analysis for high molecular weight proteins should also aim to reduce the risk of missing important tertiary structure information and also focus on the preservations of native structure.

From the SEC-triple detection experiments, due to time limitations, no structural information were revealed. However, from these experiments it was clear that for further research a column with better separation at the lower molecular weight range is necessary. A possible recommendation could be the Tosoh TSKgel G3000SWXL column that has bigger pore size than the current column used. Having a more suitable column the SEC-triple detection experiments of the three examined buffers might be able to reveal structural / shape differences in solution.

Last but not least for L-asparaginase experiments the assumptions derived from the CE experiments related to the denaturation products upon stress with sodium hydroxide and ammonium bicarbonate could be verified with SEC-MS, except of the deamidation. The initial CE experiments for asparaginase stressed with sodium hydroxide revealed three different products, the tetrameric, monomeric species and possible deamination. From SEC-MS the presence of the tetrameric and the increase of the monomeric species in time was observed. From the stress upon ammonium bicarbonate, despite the three negatively charged species present in CE, with SEC two peaks were observed that could be assigned to high molecular weight aggregates and the tetrameric species. However, under SEC-MS only the higher molecular weight species could not be identified due to their molecular weight and low abundance.

REFERENCES

Arakawa, T. E. (2010). The critical role of mobile phase composition in size exclusion chromatography of protein pharmaceuticals. *Journal of pharmaceutical sciences*, 99(4), 1674-1692.

Armenta, S. A. (2011). A review of recent, unconventional applications of ion mobility spectrometry (IMS). *Analytica chimica acta*, 703(2), 114-123.

Benesch, J. L. (Chemical reviews). Protein complexes in the gas phase: technology for structural genomics and proteomics. *2007*, 107(8), 3544-3567.

Brutscher, B. B. (1997). Backbone dynamics and structural characterization of the partially folded A state of ubiquitin by 1H, 13C, and 15N nuclear magnetic resonance spectroscopy. *Biochemistry*, 36(42), 13043-13053.

Cassou, C. A. (2013). Electrothermal supercharging in mass spectrometry and tandem mass spectrometry of native proteins. *Analytical chemistry*, 85(1), 138.

Cassou, C. A. (2014). Anions in electrothermal supercharging of proteins with electrospray ionization follow a reverse Hofmeister series. *Analytical chemistry, 86(3), 1640-1647.*

Charlotte. (July 2017). Amsterdam, the Netherlands: Vrije University.

Clarke, P. (2003). Determination of molecular size in polymers and proteins by GPC/SEC. *LC GC EUROPE*, 42-43.

Cumeras, R. F. (2015). Review on ion mobility spectrometry. Part 1: current instrumentation. *Analyst*, 140(5), 1376-1390.

Dokland, T. (2009). Back to the basics: The fundamentals of cryo-electron microscopy. *Microscopy and Microanalysis*, 15(S2), 1538-1539.

Duc, N. M. (2015). Effective application of bicelles for conformational analysis of g protein-coupled receptors by hydrogen/deuterium exchange mass spectrometry. . *Journal of The American Society for Mass Spectrometry*, 26(5), 808-817.

Fekete, S. B. (2014). Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *Journal of pharmaceutical and biomedical analysis*, 101, 161-173.

Feng, R. &. (1993). Stepwise refolding of acid-denatured myoglobin: Evidence from electrospray mass spectrometry. *Journal of the American Society for Mass Spectrometry, 4(8), 638-645.*

Fernandez-Lima, F. A. (2011). Note: Integration of trapped ion mobility spectrometry with mass spectrometry. *Review of Scientific Instruments*, 82(12), 126106.

Fernández-Maestre, R. (2012). Ion mobility spectrometry: history, characteristics and applications. *Revista UDCA Actualidad & Divulgación Científica*, 15(2), 467-479.

García, M. C. (2005, October). The effect of the mobile phase additives on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B, 825*(2), 111-123. doi:10.1016/j.jchromb.2005.03.041

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature*, 426(6968), 895-899.

Greenfield, N. J. (2006). Using circular dichroism spectra to estimate protein secondary structure. *Nature protocols*, 1(6), 2876-2890.

Guttman, M. &. (2016). Chapter Fifteen-Isotope Labeling of Biomolecules: Structural Analysis of Viruses by HDX-MS. *Methods in enzymology*, 566, 405-426.

Han, L. H. (2013). Han, L., HyDramatically stabilizing multiprotein complex structure in the absence of bulk water using tuned Hofmeister salts. *Faraday discussions*, 160, 371-388.

Hardy, J. G. (2008). Polymeric materials based on silk proteins. *Polymer*, 49(20), 4309-4327.

Hauschild, A. C. (2012). Computational methods for metabolomic data analysis of ion mobility spectrometry data—reviewing the state of the art. *Metabolites*, 2(4), 733-755.

Heck, A. J. (2008). Native mass spectrometry: a bridge between interactomics and structural biology. *Nature methods*, 5(11), 927.

Hedge, J. B. (2013, May). Effects of Ammonium Bicarbonate on the Electrospray Mass Spectra of Proteins: Evidence for Bubble-Induced Unfolding. *Analytical Chemistry, 85(13), 6469-6476. doi:10.1021/ac401020s*.

Hedges, J. B. (2013). Effects of ammonium bicarbonate on the electrospray mass spectra of proteins: evidence for bubble-induced unfolding. . *Analytical chemistry*, 85(13), 6469-6476.

Hernandez, D. R.-L. (2014). Ion dynamics in a trapped ion mobility spectrometer. *Analyst*, 139(8), 1913-1921.

Hong, P. K. (2012). A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *Journal of liquid chromatography & related technologies*, 35(20), 2923-2950.

Hoskins, J. N. (2011). Architectural differentiation of linear and cyclic polymeric isomers by ion mobility spectrometry-mass spectrometry. *Macromolecules*, 44(17), 6915-6918.

Kanu, A. B. (2008). Ion mobility–mass spectrometry. *Journal of Mass Spectrometry*, 43(1), 1-22.

Kebarle, P. &. (2009). Electrospray: from ions in solution to ions in the gas phase, what we know now. *Mass spectrometry reviews*, 28(6), 898-917.

Konermann, L. &. (1998). Unfolding of proteins monitored by electrospray ionization mass spectrometry: a comparison of positive and negative ion modes. *Journal of the American Society for Mass Spectrometry*, 9(12), 1248-1254.

Konijnenberg, A. B. (2013). Native ion mobility-mass spectrometry and related methods in structural biology. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1834(6), 1239-1256.

Koning, C. d. (August 2015). *Development of a capillary zone electrophoresis method to characterize the ALL drug L-asparaginase.* Amsterdam, The Netherlands: Vrije University.

Kopaciewicz, W., & Regnier, F. E. (1982). Nonideal size-exclusion chromatography of proteins: Effects of pH at low ionic strength. *Analytical Biochemistry, 126*(1), 8-16. doi:10.1016/0003-2697(82)90102-6

Kostanski, L. K. (2004). Size-exclusion chromatography—a review of calibration methodologies. *Journal of biochemical and biophysical methods*, 58(2), 159-186.

Kükrer, B. e. (2010). "Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography.". *Pharmaceutical research 27.10 (2010): 2197-2204.*

Lamb, J. K. (2008). Reconstructing three-dimensional shape envelopes from time-resolved small-angle X-ray scattering data. *Journal of applied crystallography*, 41(6), 1046-1052.

Lanucara, F. H. (2014). The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. *Nature chemistry*, 6(4), 281-294.

Lemaire, D. M. (2001). Stabilization of gas-phase noncovalent macromolecular complexes in electrospray mass spectrometry using aqueous triethylammonium bicarbonate buffer. *Analytical chemistry*, 73(8), 1699-1706.

Leney, A. C. (2017). Native Mass Spectrometry: What is in the Name? *Journal of The American Society for Mass Spectrometry*, 28(1), 5-13.

Lifshitz, C. &. (2006). *Principles of mass spectrometry applied to biomolecules (Vol. 10).* John Wiley & Sons.

Link, G. W. (1985). Effects of solutions used for storage of size-exclusion columns on subsequent chromatography of peptides and proteins. *Journal of Chromatography A.*, 331, 253-264.

Liu, F. C. (2016). On the structural denaturation of biological analytes in trapped ion mobility spectrometry–mass spectrometry. *Analyst*, 141(12), 3722-3730.

Lo Nostro, P. &. (2012). Hofmeister phenomena: an update on ion specificity in biology. . *Chemical reviews, ,* 112(4), 2286-2322.

Malheiro, D. (2016). *The effect of mobile phase additives on the separation and ionization of macromolecules with aqueous size-exclusion chromatography- electrospray ionization mass spectrometry.* Vrije University, Division of BioAnalytical Chemistry, Amsterdam, Netherlands.

Marcoux, J. &. (2013). Twenty years of gas phase structural biology. *Structure*, 21(9), 1541-1550.

May, J. C. (2015). Ion mobility-mass spectrometry: time-dispersive instrumentation. *Anal. Chem*, 87(3), 1422-1436.

Mertens, H. D. (2010). Structural characterization of proteins and complexes using small-angle X-ray solution scattering. *Journal of structural biology*, 172(1), 128-141.

Michelmann, K. S. (2015). Fundamentals of trapped ion mobility spectrometry. *Journal of The American Society for Mass Spectrometry*, 26(1), 14-24.

Nakanishi, K. S. (2001). On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. . *Journal of Bioscience and Bioengineering*, 91(3), 233-244.

Násztor, Z. B. (2016). The interfacial tension concept, as revealed by fluctuations. *Current Opinion in Colloid & Interface Science*, 23, 29-40.

Násztor, Z. B. (2016). The interfacial tension concept, as revealed by fluctuations. *Current Opinion in Colloid & Interface Science*, 23, 29-40.

Neya, S. F. (1993). Structural analysis of the myoglobin reconstituted with iron porphine. *Journal of Biological Chemistry*, 268(12), 8935-8942.

Porta, T. V. (2013). Gas-phase separation of drugs and metabolites using modifier-assisted differential ion mobility spectrometry hyphenated to liquid extraction surface analysis and mass spectrometry. *Analytical chemistry*, 85(24), 11771-11779.

Prakash, H. K. (2010). Effects of salts on the charge-state distribution and the structural basis of the most-intense charge-state of the gaseous protein ions produced by electrospray ionization. *International Journal of Mass Spectrometry*, 289(2), 84-91.

Rabiller-Baudry, M. &. (2001). Specific adsorption of phosphate ions on proteins evidenced by capillary electrophoresis and reversed-phase high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, 753(1), 67-77.

Record, M. T. (2013). Introductory lecture: Interpreting and predicting Hofmeister salt ion and solute effects on biopolymer and model processes using the solute partitioning model. . *Faraday discussions*, 160, 9-44.

Ridgeway, M. E. (2016). Gated trapped ion mobility spectrometry coupled to fourier transform ion cyclotron resonance mass spectrometry. *International Journal for Ion Mobility Spectrometry*, 19(2-3), 77-85.

Ruotolo, B. T. (2007). Ion Mobility–Mass Spectrometry Reveals Long-Lived, Unfolded Intermediates in the Dissociation of Protein Complexes. *Angewandte Chemie International Edition*, 46(42), 8001-8004.

Šamalikova, M. M. (2004). Interpreting conformational effects in protein nano-ESI-MS spectra. *Analytical and bioanalytical chemistry*, 378(4), 1112-1123.

Sterling , H. J., Batchelor, J. D., Wemmer, D. E., & Williams, E. R. (2010, June). Effects of Buffer Loading for Electrospray Ionization Mass Spectrometry a Noncovalent Protein Complex that Requires High Concentration of Essential Salts. *American Society for Mass Spectrometry, 21*(6), 1045-1049. doi:10.1016/j.jasms.2010.02.003.

Steven, A. C. (2008). The future is hybrid. . *Journal of structural biology.*, 163(3), 186-195.

Stutz, H. (2009). Protein attachment onto silica surfaces–a survey of molecular fundamentals, resulting effects and novel preventive strategies in CE. . *Electrophoresis*, 30(12), 2032-2061.

Tayyab, S. Q. (1991). Size exclusion chromatography and size exclusion HPLC of proteins . *Biochemical education*, 19(3), 149-152.

Tumolo, T. A. (2004). Determination of the refractive index increment (dn/dc) of molecule and macromolecule solutions by surface plasmon resonance. *Analytical biochemistry*, 333(2), 273-279.

Uetrecht, C. V. (2008). Stability and shape of hepatitis B virus capsids in vacuo. . *Angewandte Chemie International Edition*, 47(33), 6247-6251.

Vogler, E. A. (1998). Structure and reactivity of water at biomaterial surfaces. *Advances in colloid and interface science*, 74(1), 69-117.

Vos, J. K. (2015). Aqueous size-exclusion chromatographic separations of intact proteins under native conditions: Effect of pressure on selectivity and efficiency. Journal of separation science. *Journal of separation science*.

Wang, G. J. (2012). Evaluation of electrospray ionization mass spectrometry as a tool for characterization of small soluble protein aggregates. *Analytical chemistry*, 84(3), 1718-1724.

Wang, G. Z. (2014). Recent advances in protein NMR spectroscopy and their implications in protein therapeutics research. *Analytical and bioanalytical chemistry*, 406(9-10), 2279-2288.

Wani, A. H. (2012). Mass spectrometry studies of protein folding. *Curr Sci*, 102(2), 245-265.

Wyttenbach, T. &. (2011). Structural stability from solution to the gas phase: native solution structure of ubiquitin survives analysis in a solvent-free ion mobility–mass spectrometry environment. *The journal of physical chemistry B*, 115(42), 12266-12275.

Zangi, R. (2009). Can salting-in/salting-out ions be classified as chaotropes/kosmotropes? *The Journal of Physical Chemistry B*, 114(1), 643-650.

Zhang, Y. &. (2006). Interactions between macromolecules and ions: the Hofmeister series. . *Current opinion in chemical biology*, 10(6), 658-663.

Zhao, H. B. (2011). On the distribution of protein refractive index increments. *Biophysical journal*, 100(9), 2309-2317.

Zhou, Z. H. (2008). Towards atomic resolution structural determination by single-particle cryo-electron microscopy. *Current opinion in structural biology*, 18(2), 218-228.

Zubarev, R. A. (2013). Orbitrap mass spectrometry. *Analytical chemistry, 85(11), 5288-5296.*

APPENDIX

APPENDIX I: COMPARISON OF DENATURED AND NATIVE MASS SPECTRUM UNDER DIFFERENT CONDITIONS

In the research of (Cassou C. A., 2014), the effects of different anions on the electrothermal supercharging of proteins were investigated based on the difference in the average and maximum charge states of nine proteins under electrospray ionization. The concluded results showed that there was a significant increase of both the maximum and averaged charge states compared to their native spectra obtained with lower spray potentials that followed the Hofmeister series over a wide range of salt concentrations.

This study was used as an example, in order to highlight the difference between a native and a denatured MS spectrum along with the effect of the anions.
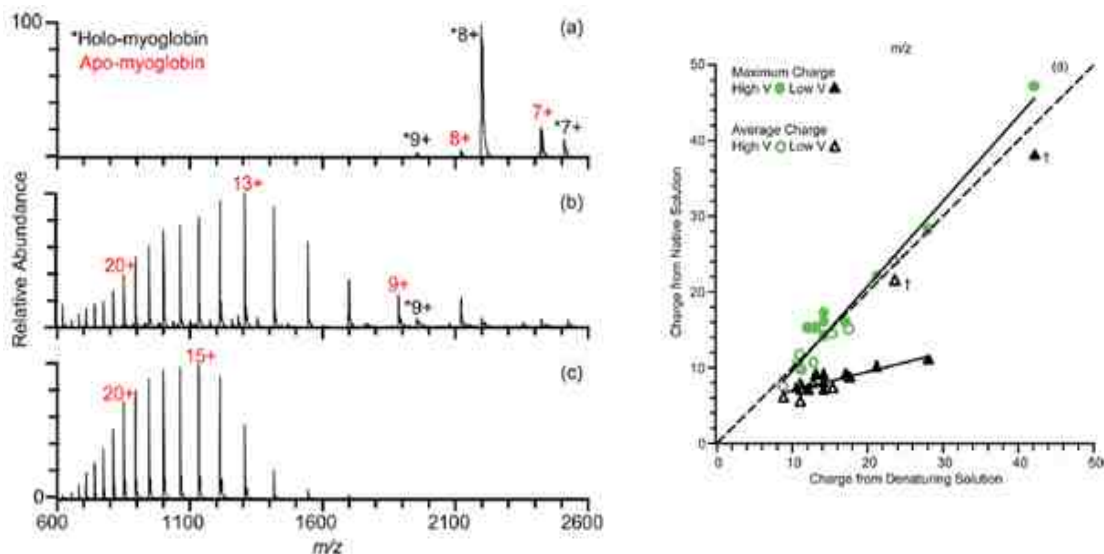
Figure 25. The monomodal and bimodal distribution of myoglobin under different electrospray and solution conditions as presented by (Cassou C. A., 2014). Myoglobin in ammonium bicarbonate and electrospray potentials a) +0.7 kV and b) +1.4 kV. b) Shows myoglobin under denaturing conditions of methanol and acetic acid. The graph shows the correlation between maximum and average charge states for the nine proteins under investigation with low electric field preserving the native conformations and high electric field that denaturation occured.

APPENDIX II: SIZE EXCLUSION CHROMATOGRAPHY CALIBRATION CURVE.

The calibration of SEC is being addressed in a dedicated sub-section. In Table 2 all the conditions all the conditions used for the calibration, that also led to the calculation of the third polynomial equation of log Mw against retention time and the final calculation of the K_d factor are presented.

Table 2. Conditions used for SEC calibration.				
Column	TSKgel G2000 SWXL	**Sample**	**Mw**	**pI**
	Column size: 7.8 mm I.D x 30 cm	**Thyroglobulin**	660 kDa	4.5
	Particle size: 5 µm			
Mobile phase	0.1 mol/L Na$_2$SO$_4$	**Albumin**	66.5 kDa	4.7
	0.05 % NaN$_3$			
	0.1 mol/ L Phosphate Buffer (pH 6.7)	**Myoglobin**	17 kDa	6.8, 7.2
Flow rate	0.8 mL/min			
Sample Volume	20 µL	**Uracil**	112 Da	-
Column Temperature	25°C			
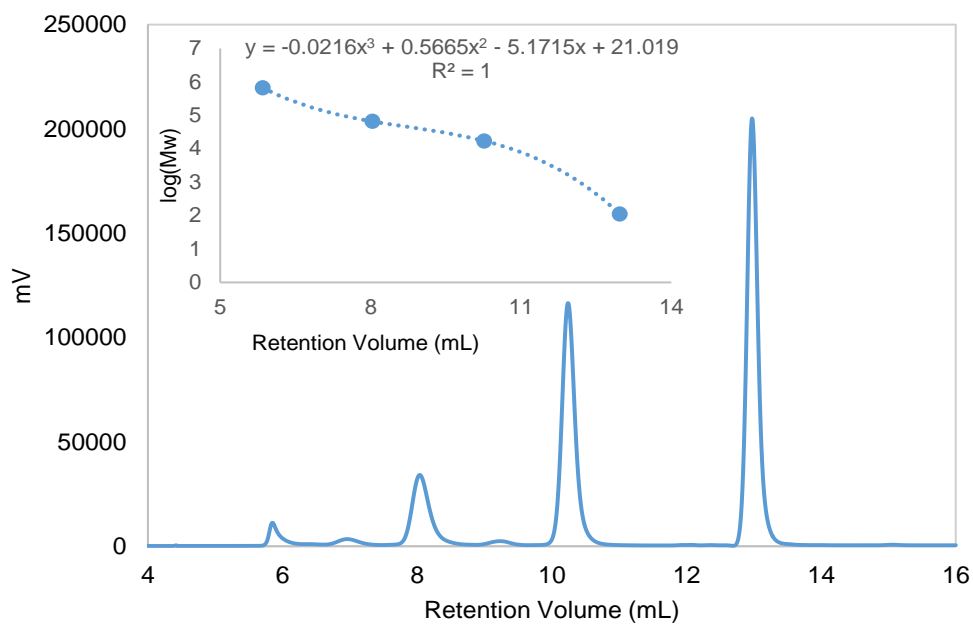
Figure 26. SEC calibration curve and equation for calculation of molecular weight

APPENDIX III: DIRECT INFUSION OF MYOGLOBIN PERFORMED BY (Malheiro, 2016)

Daniel Malheiro is a former student, worked on the same project as part of his BSc graduation project. During his study he performed direct infusion experiments of myoglobin under different conditions of the examined volatile buffers. In detail salt concentrations in the range of (10, 25, 50, 100 and 200 mM) and pH's (pH 5.9, 6.9 and 7.5) were examined. For simplicity, mass spectra of 10 and 200 mM are shown here and only for the pHs that reflect the better SEC separation conditions. These results are comparable with the results obtained with ESI-TIMS-MS, that are being discussed in this study at the section Direct Infusion Experiments at different salts and concentrations
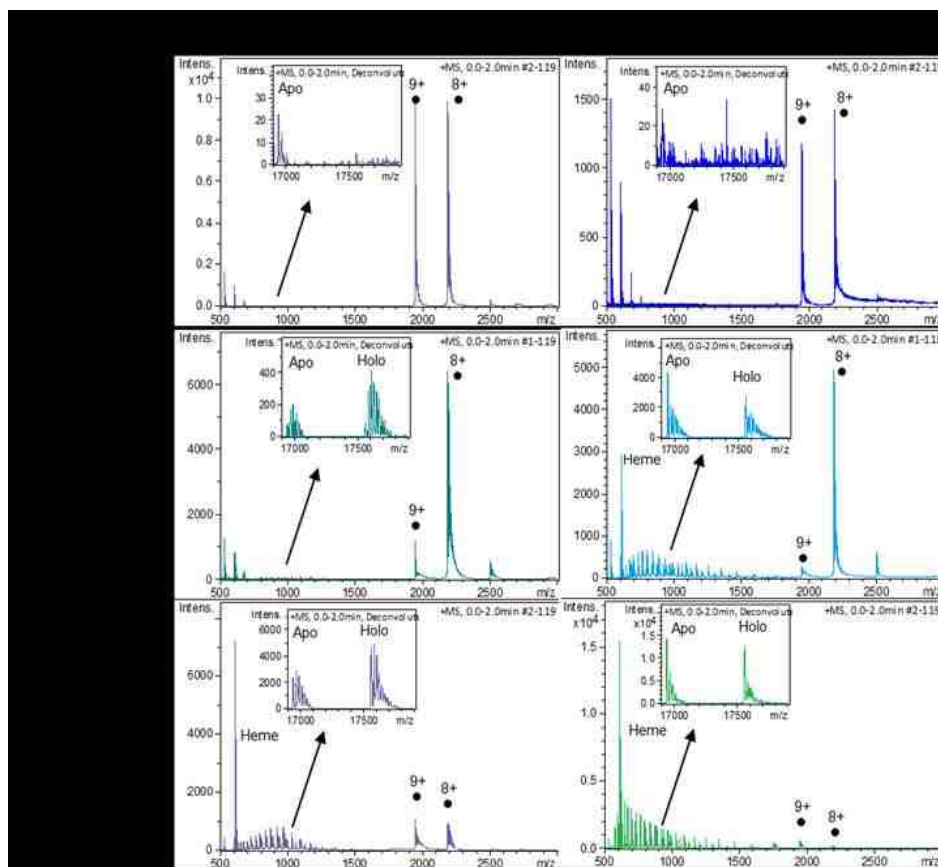
Figure 27. Direct infusion of myoglobin in a microTOF instrument for the investigation of the ionization behavior of this this analyte under different volatile salts.

APPENDIX IV: SEC-MS EXPERIMENTS OF PROTEIN MIXTURE ANALYSED WITH AMMONIUM FORMATE AND BICARBONATE

For simplicity reasons in Section SEC-UV-MS of Biomolecules not the whole data set is presented, but only the protein sample analysed with ammonium acetate. Here the results obtained with the other examined volatile salts, ammonium formate (a. 200 mM, 10 mM at pH 7.5) and ammonium bicarbonate (b. 200 mM, 10 mM at pH 7.5) are presented. The denaturation effect due to the anion and the concentration can be observed from the spectra comparison. The m/z region between 500-1400 can be considered as the denatured region, whereas m/z region between 1500-2200 is considered as the native region of myoglobin.
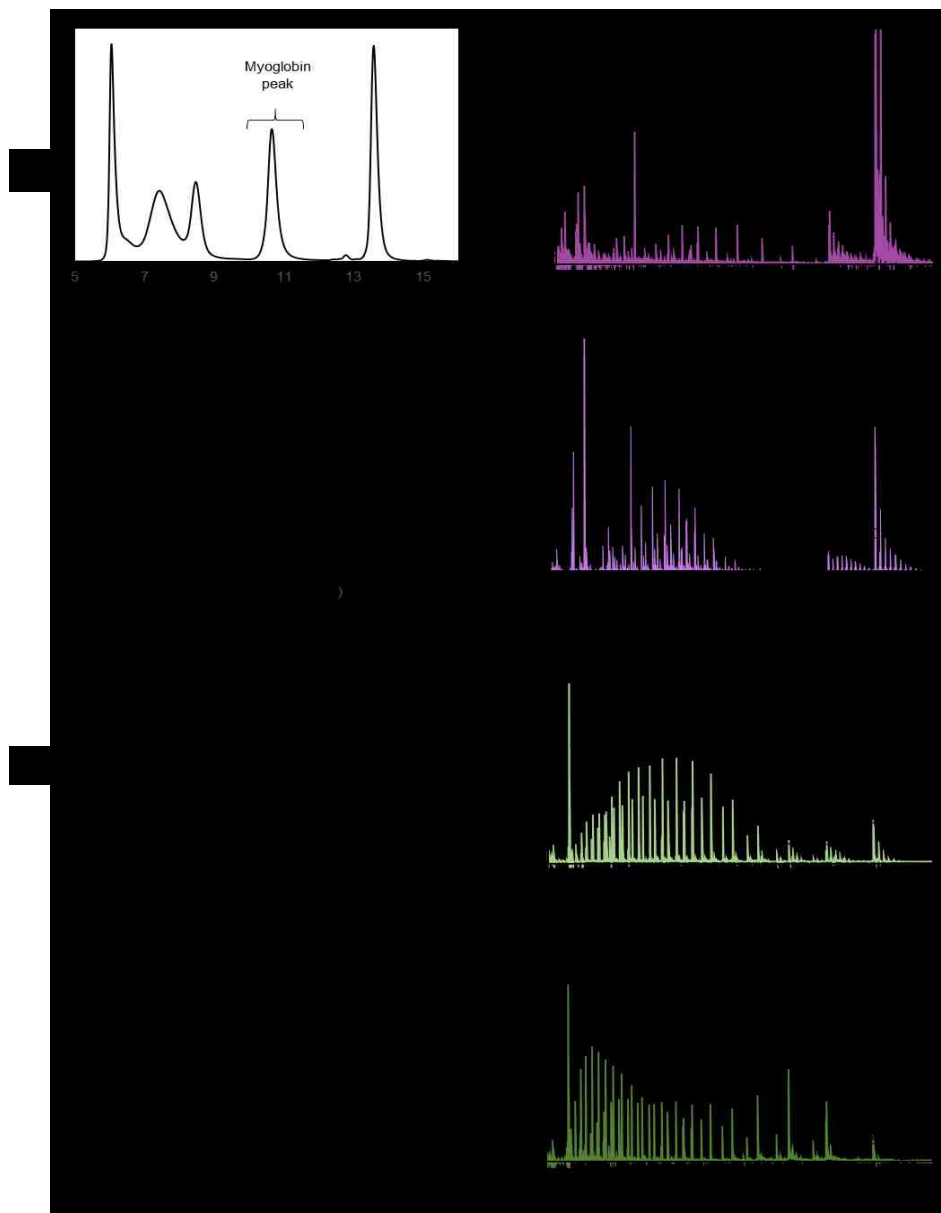
Figure 28. SEC-MS experiments of the protein mixture with a. ammonium formate and b. ammonium bicarbonate at low and high ionic strength (200 mM and 10 mM) at pH 7.5. The spectra represent only myoglobin.

APPENDIX V: DIRECT INFUSION TIMS FOR CORRELATION OF INCUBATION TIME AND DEGREE OF UNFOLDING

Direct infusion experiments under the different salts were performed in order to investigate the effect of incubation time on the degree of unfolding. For the comparison of the MS spectra and the deconvoluted spectra obtained after 5 min and 25 min no obvious difference can be assigned. As a result the contribution of incubation time in the denaturation of myoglobin was considered negligible.
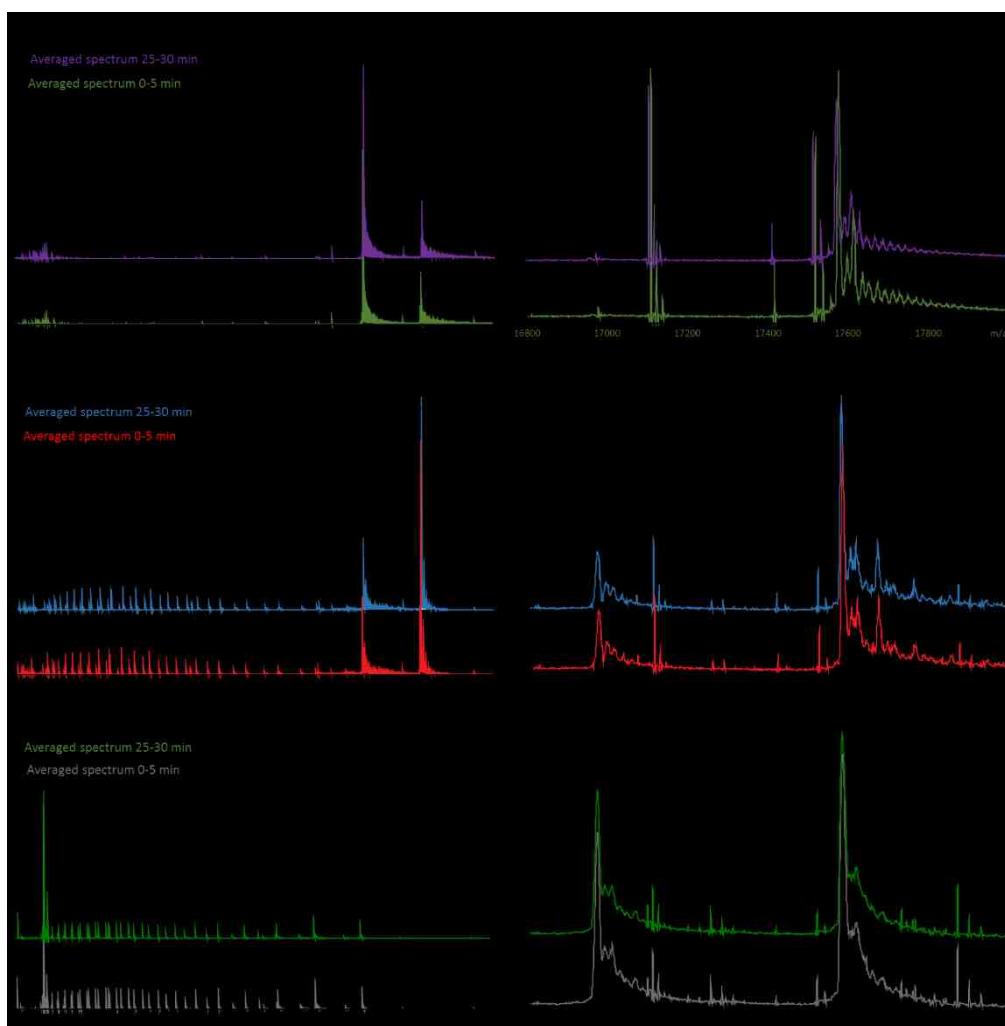

Figure 29. Direct infusion of myoglobin in ammonium acetate, formate, bicarbonate 200 mM after incubation in the buffer solution on t0= 0-5 min and t1=25-30 min. The denaturation profile of myoglobin is not related to the incubation time.

*The tune mix present in the spectra has been removed.

APPENDIX VI: TRIPLE DETECTION CALIBRATION STANDARDS ANALYZED WITH HIGH IONIC STRENGTH OF AMMONIUM BUFFER

Enabling the calculation of sizes requires a triple calibration with a narrow and broad standard. For the calibration of the triple detection broad and narrow calibration standards were analyzer with 200 mM ammonium acetate, pH 6.9 at flow rate 0.8 mL/min. Both PEG and Dextrans were dissolved in water, in order to minimize any dn/dc changes in different eluents. From the following results we can clearly see that especially for PEO no significant signal could be observed, with only indication about the elution of the PEO standards the small viscosity signal. Dextrans are eluting and could potential be used for further calculations.
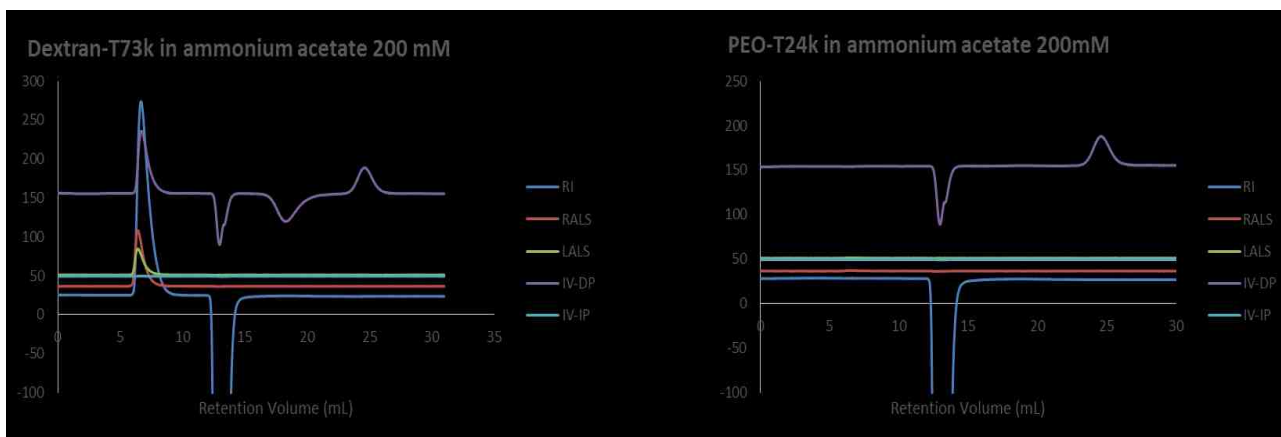

Figure 30. SEC-Triple detection experiments on high ionic strength with (left) Dextrans as the broad calibration standard and (right) PEO-T24k as the narrow calibration standards. No accurate conclusions or calculations can be drawn since the narrow calibration standards do not provide any significant signal, most likely due to agglomeration or adsorption on the column.

APPENDIX VII: TRIPLE DETECTION EXPERIMENT OF MYOGLOBIN

Triple detection experiments of myoglobin were also performed with 200 mM and 10 mM ammonium acetate at pH 6.9. Myoglobin show a strong RALS signal and small viscosity index signal, confirming its dense structure. However, it elutes very close to the total permeation (~ 11 mL) and that indicates that a better separation is necessary for radius calculations. There are also some aggregates (6 ml) seen on the RALS (no visco /RI signal). This might be an effect of non-ideal SEC conditions.

Figure 31. Myoglobin with SEC-Triple detection experiments under high and low ionic strength.
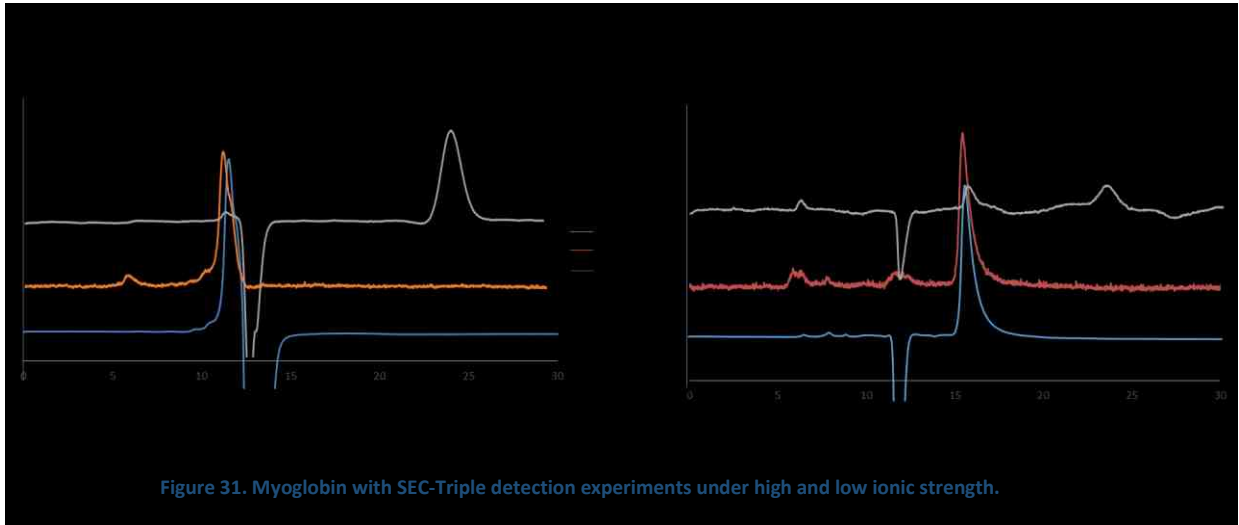
APPENDIX VIII: SIZE EXCLUSION CHROMATOGRAPHY OF L-ASPARAGINASE (Koning, August 2015).

This experiment was performed by a former BSc student as part a research related to the analysis of L-asparaginase with capillary zone electrophoresis. For the clarification of the CE results SEC was used. L-asparaginase 1 mg/mL was first analysed under native-like conditions with 50 mM ammonium formate, pH 7.0. Two peaks are observed that can be assigned as the tetramer species of L-asparaginase and higher molecular weight aggregates. For the stressing of L-asparaginase 10 mM NaOH were used. The injection volume used for these experiments was 10 µL and a flow rate of 3.5 mL/min and six consecutive runs were performed. The obtained results show inefficient separation and non-ideal SEC behavior and as a result no accurate conclusions can be drawn.
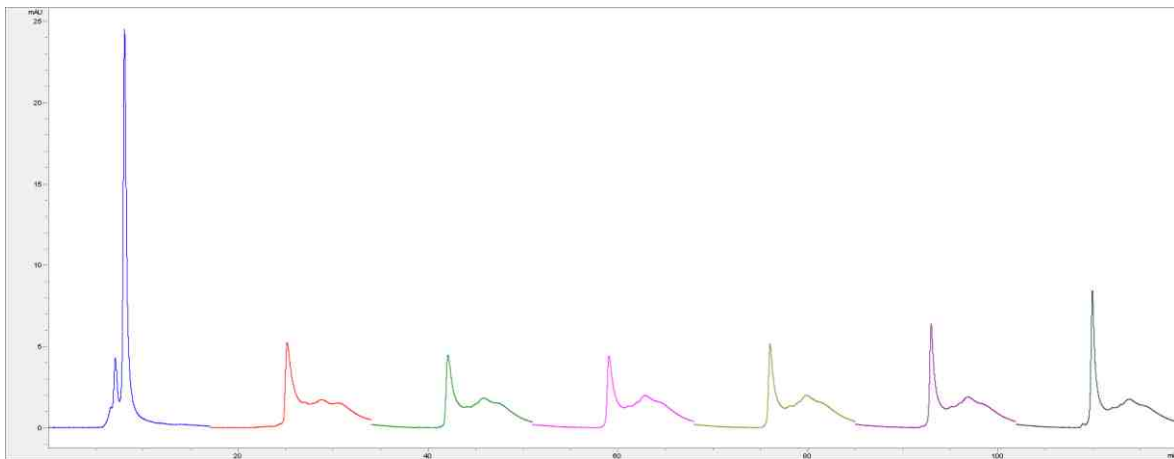

Figure 32. SEC-UV spectra of asparaginase. under stress with 10 mM NaOH. Consecutive run was injected 17 minutes after the previous run. The blue trace represents the non stressed-native Asparaginase in aqueous solution. The main most intense peak display the same retention time as the main peak under native conditions, which corresponds to the tetrameric structure.

APPENDIX IX: CE EXPERIMENTS OF L-ASPARAGINASE TREATED WITH SODIUM HYDROXIDE. (Goethals, July 2017)

L-asparaginase is treated with 10 mM NaOH and measured in 20 concecutive runs with CE-UV. The analysis was performed with a BGE of 400 mM Trizma®base and 400mM boric acid at pH 8.38. An increase of the three assigned peaks can be observed (A, B, C). The third impurity peak becomes detectable after 157 minutes (run 7). The results indicate that these impurities are either small molecules or positively charged, a hypothesis that was also investigated with SEC-MS experiments. Peak A includes the monomeric species formed by the degradation, whereas B and C are deamidated variants of the monomers.
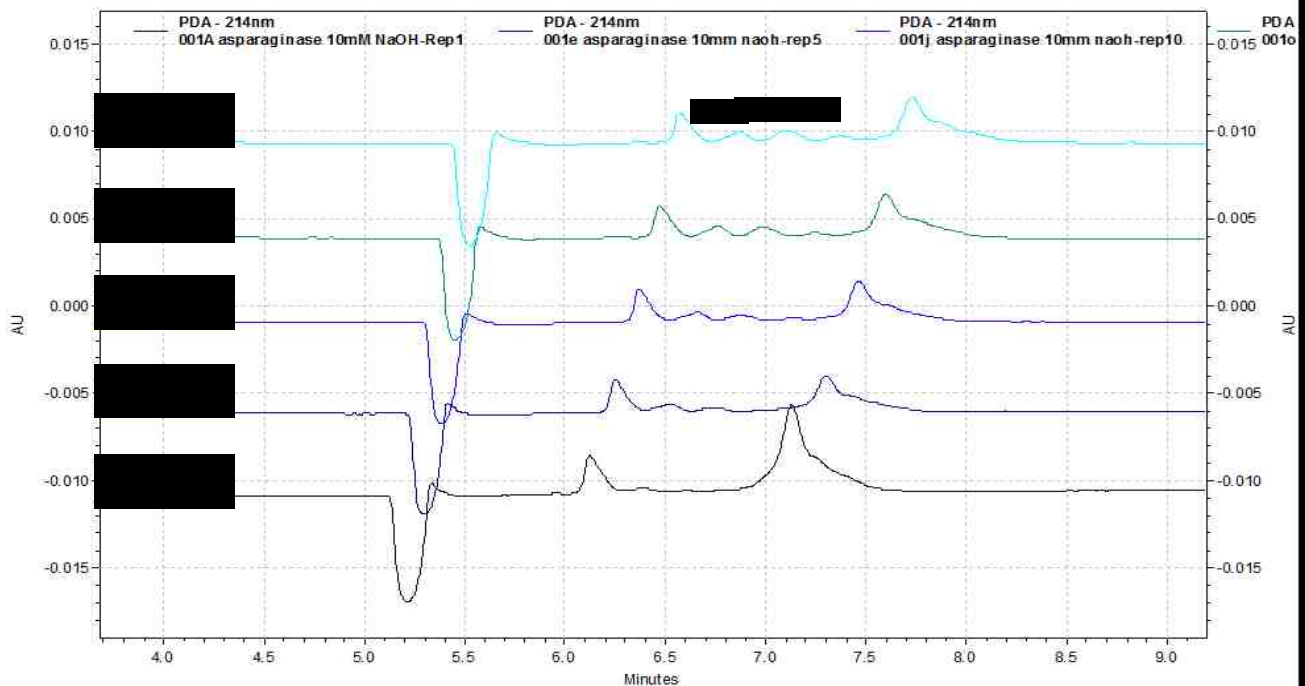

Figure 33. CE of L-asparaginase stressed with 10 mM NaOH. 20 conceqvtive runs were performed for monitoring the increase of the A,B,C impurity peaks. All of the impurity peaks are migrating earlier, so either they are smaller molecular weight compounds or more positively charged. This hypothesis was proved also with SEC-MS experiments, in order to prove that peak A is due to degradation of the tetramer species into the monomers and peaks B and C are due to deamidation of these monomers.

APPENDIX X: CE EXPERIMENTS OF L-ASPARAGINASE TREATED WITH AMMONIUM BICARBONATE (Goethals, July 2017).

L-Asparaginase was treated with 1% NH_4HCO_3 for 24 h. The CE analysis was conducted after 0, 4, 8, 16, 20, 24 h using a BGE of 400 mM Trizma®base and 400mM boric acid at pH 8.38. As illustrated in Figure 33 peak A exists from the very first injection of 0 h of stress and has a longer migration time in comparison to the main peak. A decrease of this peak while peaks B and C start existing, in combination to the fact that they have a longer migration time indicate that these might be deamidated more negatively charged species. Again the hypothesis for the nature of these peaks was meant to be identified with comparative experiments of SEC.
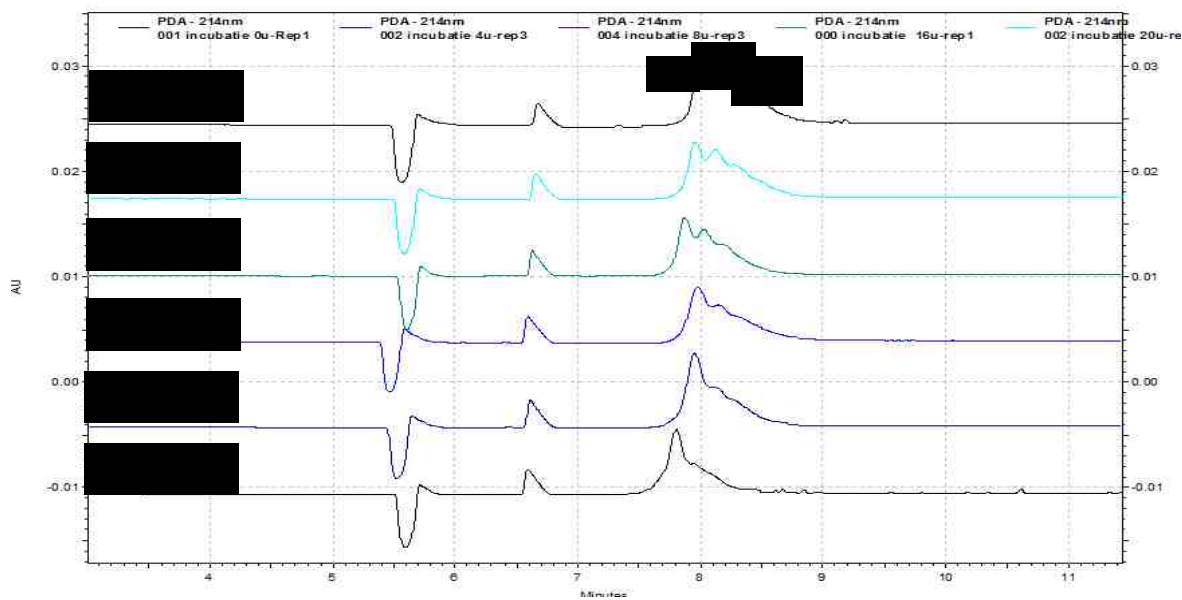

Figure 34. CE of L-asparaginase stressed with 1% w/v ammonium bicarbonate (NH₄HCO₃). The stress of an asparaginase sample was monitored for 24 h and the increase of peaks A, B, C was observed. Deamidation is the most propable reaction that leads to the production of the impurity species, which leads to extra negative charge and longer migration time. In order to prove this assumption SEC-MS was performed. The conditions used for the analysis of L-asparaginase with CE were optimized and as BGE: 400mM Trizma®base and 400mM boric acid (pH 8.38) was used.

APPENDIX XI: SEC CHROMATOGRAMS OF L-ASPARAGINASE STRESSED UNDER BASIC CONDITIONS

a. Stress with 1% NH_4HCO_3: SEC-UV analysis after 0, 4, 16, 20, 24 hours. The mobile phase used for the non volatile conditions was 100 mM phosphate buffer at pH 6.9, whereas for volatile MS compatible mobile phase 200 mM ammonium acetate pH 6.9 was used. No obvious formation of lower molecular weight species was observed, whereas a slight difference in the higher molecular weight aggregates was observed between the different mobile phases.

b. Stress with 10 mM NaOH: Consecutive runs were performed with SEC-UV at a total incubation time of 150 min. The same as the above mentioned mobile phases were used. As discussed also in the respective section a different ratio between the tetrameric species and the formed monomeric species was observed according to the used mobile phase.
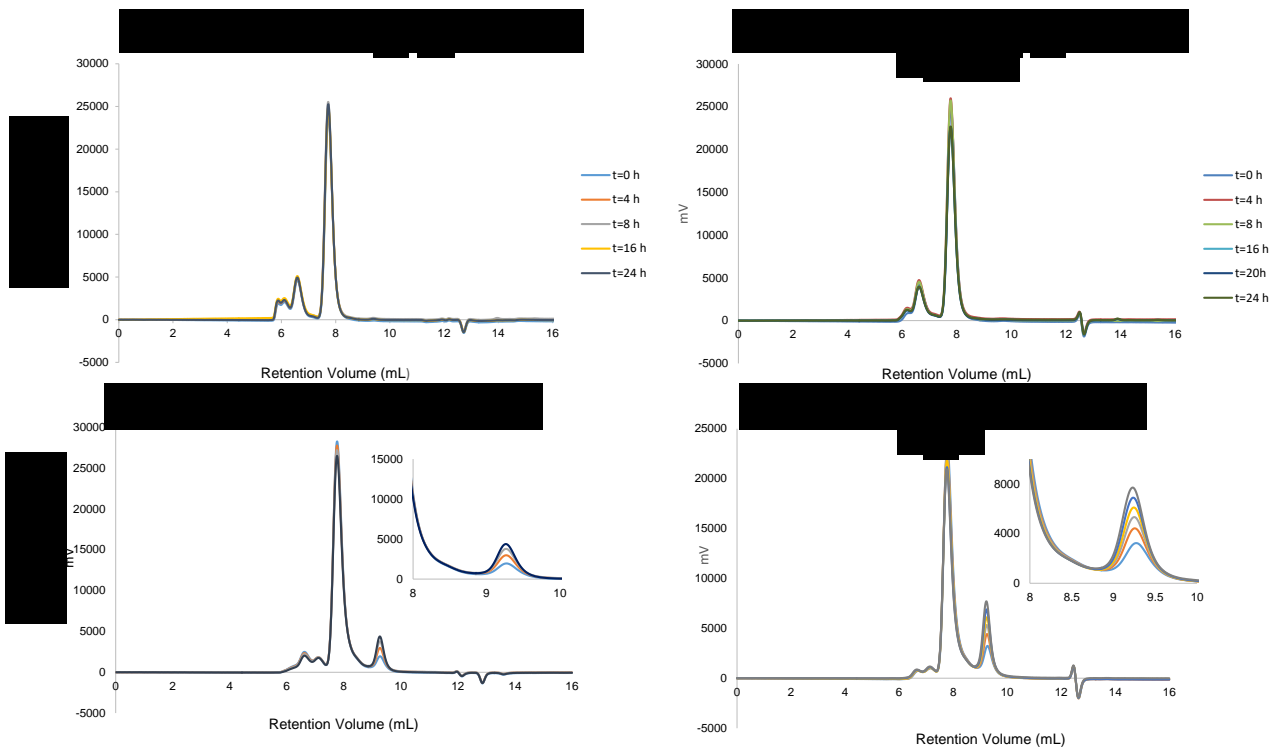

Figure 35. Chromatograms of stressed L-asparaginase in time. a. Stress of L-asparaginase with ammonium bicarbonate from 0h until 24h. b. Concecutive chromatograms during the stress of L-asparaginase with sodium hydroxide

APPENDIX XII: SEC-MS RESULTS OF L-ASPARAGINASE TREATED WITH AMMONIUM BICARBONATE

Upon stress with 1% NH_4HCO_3 and analysis with 200 mM ammonium acetate pH 6.9, no obvious deamidation could be observed. From the decomvoluted spectrum only the presence of the tetrameric species with Mw ~ 140 kDa can be identified.
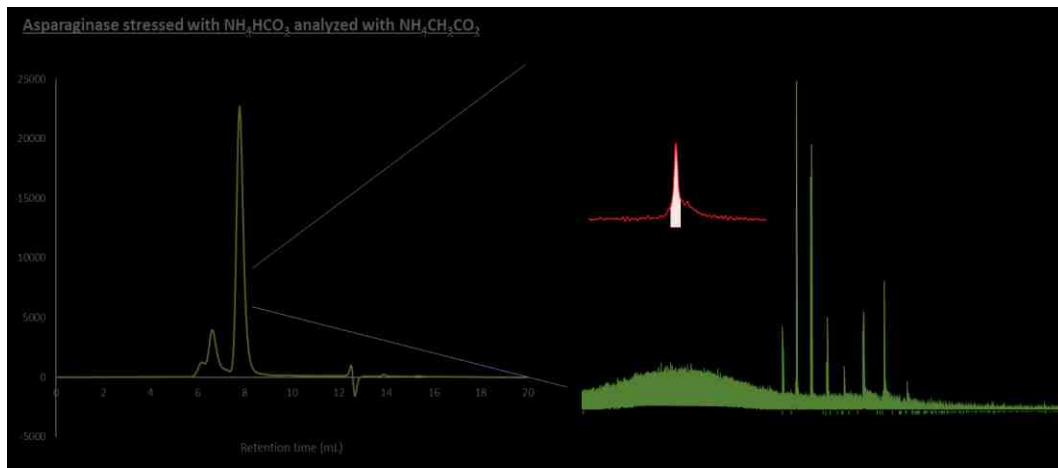

Figure 36. SEC-MS experiment of L-asparaginase stressed with ammonium bicarbonate and analyzed with ammonium acetate 200 mM, pH 6.9.

APPENDIX XIII: STRUCTURAL DENATURATION OF UBIQUITIN WITH TRAPPED ION MOBILITY MASS SPECTROMETRY (Liu, 2016)

The research of Liu shows that the extent of structural denaturation in ubiquitin ions depends significantly on the amount of the kinetic energy than an ion can gain during analysis, which is strongly related to the following parameters: DC and RF electric fields, accumulation and trapping time. In their paper DC potential difference between the ion deflector and entrance funnel was set from 30 V up to 150 V and the RF amplitude from 150 up to 230 Vpp and the following results
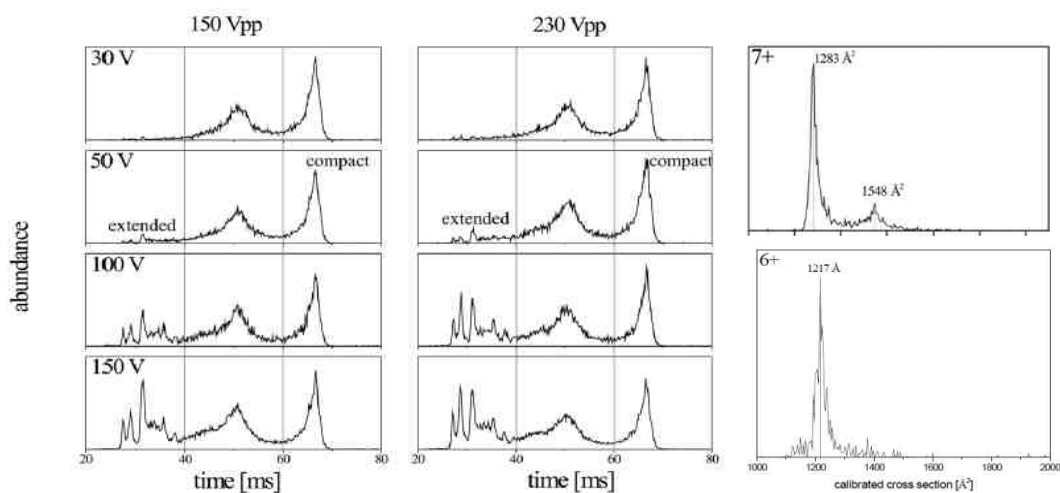

Figure 37. The effects on the denaturation of ubiquitin under varying DC potential difference between deflector and entrance funnel and the amplitude of the RF electric field were investigsted by (Liu, 2016). The amplitude RF elecetic field seems to have a minor effect on the conformations of 7+. On the right the colissional cross section distributions of 7+ and 6+ are depicted, proving two and one conformation under the examined conditions, respectively.