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Diplomarbeit

Method development for quantifying protein modifications using liquid chromatography hyphenated to tandem mass spectrometry

Ausgeführt am

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Abstract

Zearalenone (ZEN) is a mycotoxin, which is produced by various species of the fungus *Fusarium,* such as *F. graminearum*, *F. equiseti* and *F.culmorum.* Since this fungus is common to grow on crops, food and feed are often contaminated with the mycotoxin.

Romer Labs developed a quick test, in form of a lateral flow device (LFD), to determine the amount of contamination on grain. An important constituent for this LFD is zearalenone – carboxymethyl oxime (ZEN-CMO) coupled to conalbumin (CON). Since it is always desirable to produce at the lowest possible cost, it is of interest whether different synthesis strategies can lead to the same results in terms of modification efficiency on the protein, while reducing various synthesis steps or chemicals in use.

Therefore the goal was to establish a liquid chromatography – tandem mass spectrometry method (LC-MS/MS) to quantify ZEN-CMO modifications introduced onto the target protein.

For detailed protein characterization, the proteins were digested and analyzed on a nano-LC-electrospray ionization (ESI)-ion trap mass spectrometer performing collision induced dissociation (CID) fragmentation for peptide identification and modification localization. In order to maximize protein sequence coverage, two proteases were tested. Immobilized trypsin was compared to a trypsin/LysC mix. Further method development for quantification was performed on an ultra high performance liquid chromatography (UPLC) – ESI – triple quadrupole instrument.

We found trypsin/LysC to perform best in terms of sequence coverage after in solution digestion and peptide desalting. It was possible to identify 27 out of 59 possible Lysine modification sites and it was found that most of the identified protein modifications were located on the protein's surface.

Furthermore, fragmentation mass spectra gave insight into fragmentation mechanisms of ZEN-CMO and ZEN-CMO modified peptides using an ion trap mass analyzer, exhibiting reporter fragment ions which were further used to develop a quantification method applying triple quadrupole MS technology.

Zusammenfassung

Zearalenon (ZEN) ist ein Mykotoxin, das von verschiedenen Pilzspezies der Gattung *Fusarium*, wie beispielsweise *F.graminearum, F. equiseti* und *F.culmorum*, produziert wird. Da dieser Pilz hauptsächlich auf Getreidepflanzen wächst, sind Lebensmittel und Futtermittel häufig mit dem Mykotoxin kontaminiert.

Aus diesem Grund entwickelte die Firma Romer Labs einen Schnelltest, mit dem die Menge an Kontamination in einer Getreideprobe festgestellt werden kann. Eine wichtige Komponente in diesem Test ist Zearalenone – Carboxymethyloxim gekoppelt an Conalbumin. Da es immer erstrebenswert ist möglichst billig zu produzieren, war es von Interesse herauszufinden, ob verschiedene Synthesestrategien eine Reduktion der verwendeten Chemikalien und der Syntheseschritte ermöglichen, und dennoch dieselbe Modifikationseffizienz am Protein erreicht werden kann.

Daher war es das Ziel eine LC-MS/MS Methode zu etablieren, die zur Quantifizierung von ZEN-CMO Modifikationen auf dem Zielprotein verwendet werden kann.

Um die Proteinkonjugate zu charakterisieren wurden sie proteolytisch abgebaut, die entstandenen Peptide auf einem nano-LC-ESI-Ionenfallen Massenspektrometer analysiert und die Lokalisierungen der Modifikationen durch dissoziations-induzierte Fragmentierung identifiziert. Um eine möglichst große Sequenzabdeckung zu erreichen wurden 2 Proteasen getestet. Immobilisiertes Trypsin und ein Trypsin/LysC Mix wurden verglichen. Die Methodenentwicklung zur Quantifizierung erfolgte auf einem „Ultra High Performance“ – Flüssigkeitschromatographie - Elektrosprayionisations -Triplequadrupol Instrument.

Durch die Verwendung eines Ionenfallen-Massenanalysators erhielt man Einblick in den Fragmentationsmechanismus von ZEN-CMO und von ZEN-CMO modifizierten Peptiden und Reporterionen wurden für die Entwicklung einer Triple-Quadrupol-MS-Quantifizierungsmethode ausgewählt.

Der In-Lösungsverdau mit Trypsin/LysC wurde im Bezug auf die Sequenzabdeckung als besser befunden. 27 von 59 Lysinen konnten als mögliche Modifikationsstellen identifiziert werden und es erscheint, dass sich die meisten Modifikationen an der Protein Oberfläche befinden.

"If we knew what it was we were doing,
it would not be called research, would it?"

Albert Einstein

Danksagung

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Abbreviations

AA	Amino acid
ACN	Acetonitrile
CE	Collision energy
CEM	Channel electron multiplier
CID	Collision induced dissociation
CON	Conalbumin
cr	coupling ratio
DTT	DL-Dithiothreitol
ESI	Electrospray ionization
FA	Formic acid
GOM	Grade of modification
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
ICR	Ioncyclotron
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LFD	Lateral flow device
LIT	Linear ion trap
LMCO	Low mass cut-off
MCP	Micro-channel plate
MRM	Multiple reaction monitoring
MS	Mass spectrometry

MS/MS	Tandem mass spectrometry
MS^n	Multi-stage mass spectrometry
m/z	Mass-to-charge-ratio
nESI	Nano electrospray ionization
nLC	Nano liquid chromatography
OT	Orbitrap
Q	Quadrupole
QIT	Quadrupole Ion Trap
QQQ	Triple quadrupole
RPC	Reversed phase chromatography
RT	Retention time
SC	Sequence coverage
SEM	Secondary electron multiplier
SIM	Single ion monitoring
S/N	Signal-to-noise-ratio
TIC	Total ion current
TOF	Time-of-flight
UHQ	Ultra high quality
UPLC	Ultra performance liquid chromatography
ZEN	Zearalenone
ZEN – CMO	Zearalenone – carboxymethyl oxime

1 Introduction

1.1 The mycotoxin zearalenone

Zearalenone (ZEN) is a secondary metabolite, which is produced by various species of the fungus *Fusarium,* such as *F. graminearum* (*Giberella zeae*), *F. equiseti* and *F.culmorum.*[1] Since these species are common to inhabit cereal plants, ZEN can often be found in wheat, barley and maize. As a result contaminations occur in food and feed.[2]

1.1.1 Physicochemical properties

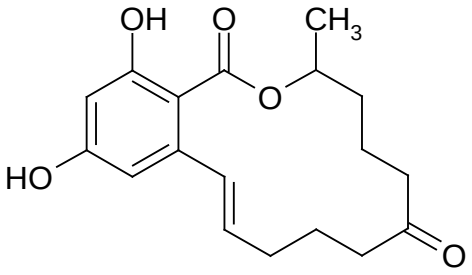


Figure 1 Zearalenone ZEN - $C_{18}H_{22}O_5$, Monoisotopic mass: 318.15 Da[i]

The chemical structure of ZEN (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-B-resorcylic acid lactone) was first determined by Urry et al. in 1966[3] (see Figure 1). The trivial name ZEN is a result of *Giberella zeae*, RAL for resorcylic acid lactone, -ene for the C-11´ to C-12´ double bond and –one, for the C-7 ketone, put together. The abbreviations ZEA or ZON are also commonly used in the literature.

ZEN is a white, crystalline substance with an elemental composition of $C_{18}H_{22}O_5$. It has a monoisotopic molecular mass of 318.15 Da. Its melting point is at 164-165°C.[3] ZEN is a hydrophobic substance and therefore insoluble in water (0.002 g / 100 g at 25°C). It is however soluble in organic solvents (24 g / 100 g in ethanol at 25°C and 8.6 g / 100 g in acetonitrile (ACN) at 25°C).[4]

[i] Chemical structures were drawn using MDL ISIS Draw 2.5

1.1.2 Toxicity of zearalenone

While ZEN has actually a low acute toxicity, it is known to have estrogenic effects due to its association with estrogen receptors.[5] Therefore it mainly causes problems with the reproductive system. Pigs seem to be especially sensitive to ZEN and are the most regularly affected species of the many domestic animals.[6] The two suggested biotransformation pathways for ZEN are hydroxylation, leading to the formation of α- and β-zearalenol, and conjugation with glucuronic acid. α-zearalenol is more estrogenic and β-zearalenol is less estrogenic than ZEN. Compared to other animal species the formation of α-zearalenol in pigs is higher. This is believed to be an explanation for the higher sensitivity of pigs to ZEN, compared to other species like chicken or cattle.[7]

Common pathological effects observed in pigs are splay-legs, swelling of the vulva, increased embryonic and fetal death[8], pseudo pregnancy, infertility[6] and changes in the serum levels of progesterone and estradiol in laboratory animals.[9] Metabolism of ZEN into α-zearalenol mainly occurs in the liver, making it a target organ.[7] ZEN can induce liver lesions, which could lead to the development of hepatocarcinoma. Changes in enzymatic parameters caused by ZEN have been observed in rats, rabbits and gilts. ZEN has been shown to enhance lipid peroxidation.[10] A study even showed that ZEN can be genotoxic in *in vitro* cultures of bovine lymphocytes.[11]

ZEN is one of the most important exponents of *Fusarium* mycotoxins in regard to animal health implications and economic losses.[12] The European Food Safety Authority estimated the chronic dietary exposure to ZEN based on the available occurrence data in 2011. According to their results the intake is below the tolerable daily intake for all age groups of humans and therefore not a health concern.[13] ZEN is often co-occurring with fumonisins and deoxynivalenol. These mycotoxins therefore might be involved in synergistic interactions.[14]

ZEN only poses a real threat to animal and human health, when it is absorbed in heavy dose over a longer period of time. Therefore further studies on the absorption, metabolism and eventual storage of ZEN are needed to evaluate its transfer rate to animal products.[2]

1.2 Lateral flow devices and the role of ZEN-CMO-CON-conjugates

1.2.1 Mycotoxin quick test

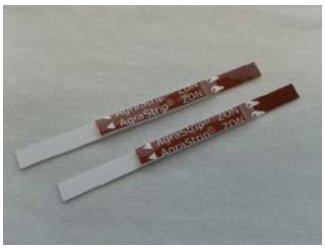


Figure 2 AgraStrip® by Romer Labs - quick test for ZEN contamination in corn samples

Since lateral flow devices (LFD) are easy to use and give quick results, they are today very commonly used as mycotoxin rapid test strips, e.g. the AgraStrip® (Romer Labs Division Holding GmbH, Tulln, Austria) for ZEN contamination (see Figure 2). LFDs make use of sample flow along a nitrocellulose membrane due to capillary forces and give qualitative as well as quantitative results. The tests are based on a competitive immunoassay that uses labeled antibodies as a signal reagent. In most cases colloidal gold is used to label antibodies, since it is commonly available and forms conjugates with antibodies very easily. Due to surface plasmon resonance effects the colloidal gold particles appear to have a red color, which is convenient for test strip signaling.[15]

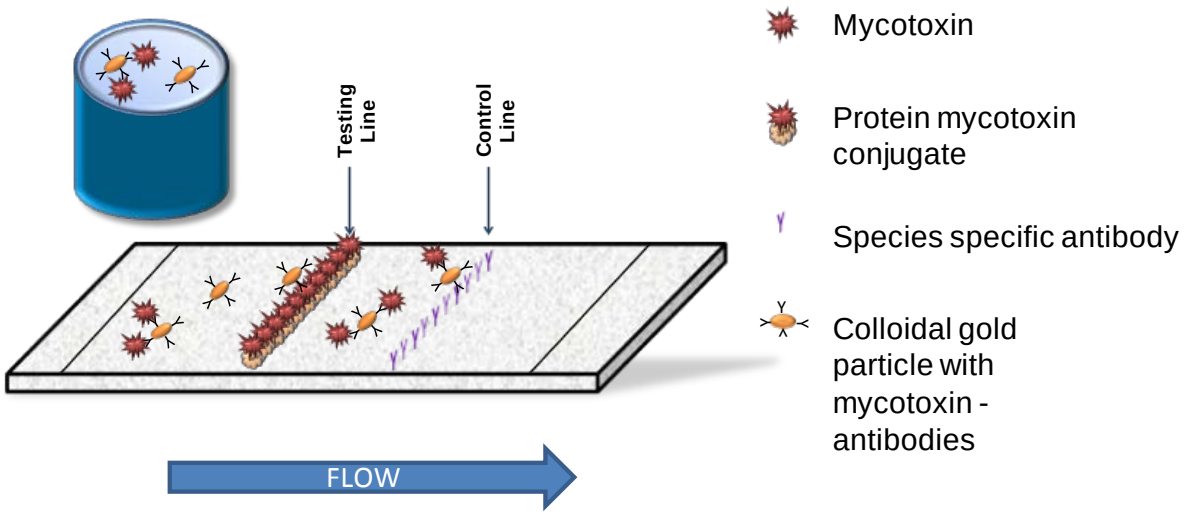


Figure 3 Composition and principle of a LFD mycotoxin quick test[15]

Other colloidal gold based LFDs have been developed for deoxynivalenol[16], aflatoxin B_1[17], fumonisin B_1[18] and T-2 toxin[19].

The composition and principle of a mycotoxin quick test is displayed in Figure 3. The testing line consists of protein-mycotoxin conjugates and the control line of species specific antibodies. Previous to the test, extraction of the corn sample has to be performed. In a micro well this extract is mixed with colloidal gold particles that are modified with mycotoxin-antibodies. During this step free mycotoxins, which contaminate the corn sample, bind to the mycotoxin-antibodies. Next, the test strip is inserted into the micro well and the content starts to migrate on the nitrocellulose membrane. At first, extracted analytes, colloidal gold particles carrying antibodies and eventually formed gold-antibody-mycotoxin conjugates encounter the testing line. At this point the colloidal gold particles, which did not yet bind to free mycotoxin in the extract and have therefore unoccupied antibodies, bind to the mycotoxin on the protein-mycotoxin conjugates and thereby concentrate to form a visible line. The colloidal gold particles, which have already bound to free mycotoxin (the contamination) in the sample extract, can no longer bind at the testing line and will move past it. This means, the more mycotoxin contamination is contained in the corn sample, the lighter colored the testing line will be. If the sample is not contaminated, the testing line will be very intense and clearly visible. The results can be quantified by comparing the color intensity of the strip to a standard series of tests with known mycotoxin concentrations by using a photometric strip reader, e.g. AgraVision™ Reader by Romer Labs (See Figure 4). All labeled colloidal gold particles are bound on the control line. This line should therefore always be visible, to confirm that the test is working correctly.[15]

Even though there are still problems in the development of LFDs, like insufficient sensitivity, selectivity and strong matrix dependency, high-quality test strips are a good way to complement classical detection methods, when rapid screening is needed.[15]

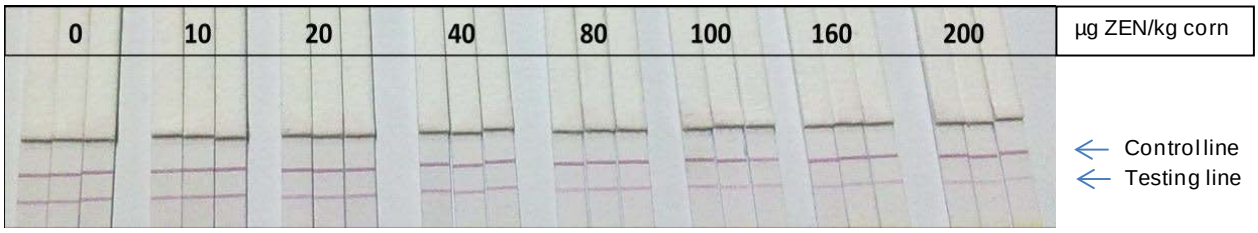


Figure 4 LFD tests showing different intensities at the testing line due to different amounts of ZEN contamination in corn samples (adapted from © Barbara Cvak, Romer Labs)

1.2.2 Protein mycotoxin conjugates

In this thesis the components of the testing line - the protein mycotoxin conjugates - were investigated. The conjugates in this study consist of zearalenone – carboxymethyl oxime (ZEN-CMO) modifications on conalbumin (CON) as carrier protein (see Figure 5).

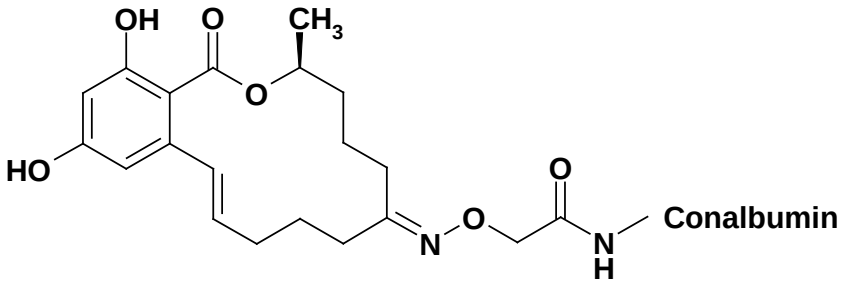


Figure 5 Structure of zearalenone – carboxymethyl oxime (ZEN-CMO) coupled to conalbumin, CON. The coupling of one ZEN-CMO molecule adds 373.15 Da (monoisotopic molecular mass) to the target molecule.

CON (see Figure 6), also known as ovotransferrin, is an iron binding transport protein, typically isolated from hen egg white. The pre-protein consists of 705 amino acids (AA) and has a molecular mass of 77777 Da. After the elimination of the signal peptide (19 AAs) the total sequence of the proteins mature form consists of 686 AAs. The protein has a total of 15 disulfide bonds and one N-glycosylation site. The protein used in this study was iron free. Information on CON was obtained from the UniProtKB database (www.uniprot.org, entry: P02789). Figure 7 shows the sequence of CON with the signal peptide still attached. For this AA sequence natural AA variations are possible. These are displayed in Table 1.

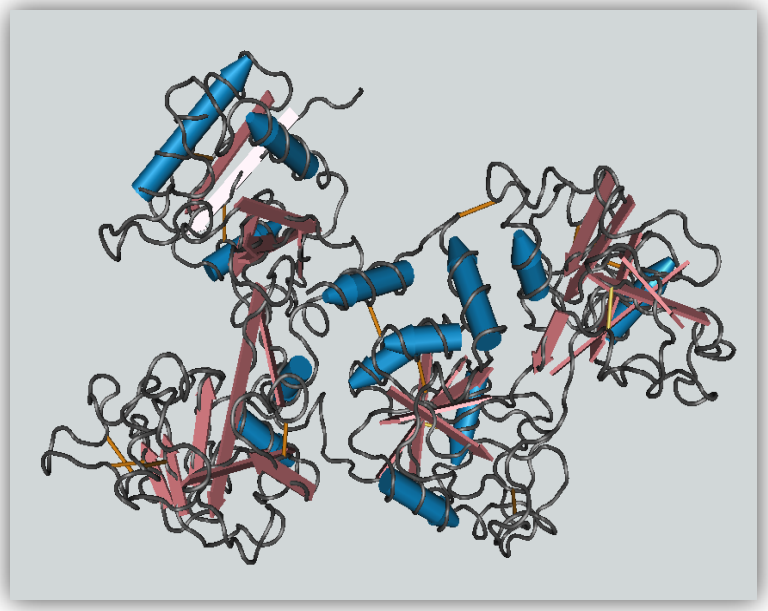


Figure 6 APO Ovotransferrin (CON) MMDB ID: 55217. PDB ID: 1AIV. Picture processed with Cn3D 4.3.1

>sp|P02789|TRFE_CHICK Ovotransferrin OS=Gallus gallus PE=1 SV=2
*MKLILCTVLSLGIAAVCFA*APPKSVIRWCTISSPEEKKCNNLRDLTQQERISLTCVQKAT
YLDCIKAIANNEADAISLDGGQAFEAGLAPYKLKPIAAEVYEHTEGSTTSYYAVAVVKKG
TEFTVNDLQGKTSCHTGLGRSAGWNIPIGTLLHRGAIEWEGIESGSVEQAVAKFFSASCV
PGATIEQKLCRQCKGDPKTKCARNAPYSGYSGAFHCLKDGKGDVAFVKHTTVNENAPDQK
DEYELLCLDGSRQPVDNYKTCNWARVAAHAVVARDDNKVEDIWSFLSKAQSDFGVDTKSD
FHLFGPPGKKDPVLKDLLFKDSAIMLKRVPSLMDSQLYLGFEYYSAIQSMRKDQLTPSPR
ENRIQWCAVGKDEKSKCDRWSVVSNGDVECTVVDETKDCIIKIMKGEADAVALDGGLVYT
AGVCGLVPVMAERYDDESQCSKTDERPASYFAVAVARKDSNVNWNNLKGKKSCHTAVGRT
AGWVIPMGLIHNRTGTCNFDEYFSEGCAPGSPPNSRLCQLCQGSGGIPPEKCVASSHEKY
FGYTGALRCLVEKGDVAFIQHSTVEENTGGKNKADWAKNLQMDDFELLCTDGRRANVMDY
RECNLAEVPTHAVVVRPEKANKIRDLLERQEKRFGVNGSEKSKFMMFESQNKDLLFKDLT
KCLFKVREGTTYKEFLGDKFYTVISSLKTCNPSDILQMCSFLEGK

Figure 7 Amino acid (AA) sequence of CON including the signal peptide in FASTA format (UniprotKB entry P02789). The signal peptide is indicated in italic letters.

Table 1 Natural AA variations for CON (UniprotKB entry P02789)

Amino acid position	Amino acid change
83	Ala -> Val
100	Val -> Ile
154	Arg -> Trp
239-240	Gln-Lys -> Leu-Asn
686	Ser -> Asn

After several tandem mass spectrometry (MS/MS) experiments, it could be concluded that the natural variation of AA 83 (Ala -> Val) occurred in the used protein. This was taken into account for further experiments (see chapter 3.2 for more information).

Since ZEN has no suitable reactive group to bind to a protein, it has to be transformed into ZEN-CMO before coupling.[20] ZEN-CMO ($C_{20}H_{25}NO_7$) has a monoisotopic molecular mass of 391.16 Da. Coupling to the protein leads to elimination of H_2O. Addition of one ZEN-CMO molecule therefore leads to an increase of the monoisotopic molecular mass of 373.15 Da for the target molecule.

As stated by Romer Labs the modification is most likely to couple to the NH_2-group of lysine (Lys), arginine (Arg) or the N-terminus. However coupling is also possible to the OH-group of serine (Ser) and threonine (Thr). For further information on coupling mechanisms see Hermanson.[21]

1.3 Aim of this study

Romer Labs produces LFDs, which can be used to determine mycotoxin contaminations in crop samples. Since the mycotoxin is the most expensive component in the production of a ZEN quick test, it was of desire to reduce the quantity of ZEN-CMO molecules needed for the production of the protein mycotoxin conjugates used in the test, while still maintaining the same amount of modifications per protein. Therefore different synthesis strategies for the protein conjugates were tested by Romer Labs. To compare these results and to have a quick and easy method to check mycotoxin-protein conjugate production batches in terms of modification efficiency, an LC-MS/MS method was required. Thus the goal of this study was to develop a method, which can be used to quantify ZEN-CMO modifications on CON.

Therefore the ZEN-CMO-CON conjugates had to be characterized and the modification localization sites were identified by performing collision induced dissociation (CID) experiments. In order to find the best suitable scan method, neutral loss scan, precursor ion scan and multiple reaction monitoring (MRM) were compared.

1.4 Sample preparation for LC-MS/MS

The most commonly used mass spectrometry (MS) - based method for studying proteins is the so called “bottom-up” approach. In this approach proteins are enzymatically or chemically cleaved into peptides prior to MS analysis.[22] It is contrary to the “top-down” approach, which is the analysis of the intact protein.[23]

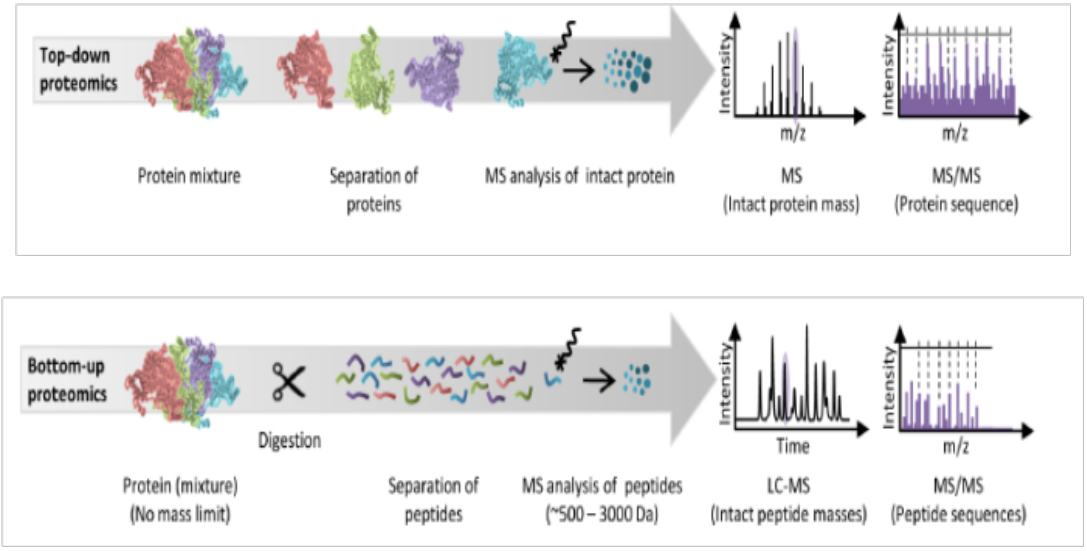


Figure 8 Strategies of top-down and bottom-up proteomics (adapted from Switzar et al.[24])

Each approach has its own advantages. An obvious advantage of the top-down approach is that sample preparation is less elaborate compared to a bottom-up approach. It also maintains information that might get lost in other approaches, like the connectivity of multiple post translational modifications.[25] However the large size of the analytes requires MS instruments with a high mass resolution and accuracy like fourier transform ion-cyclotron resonance and orbitrap instruments. The approach used to be limited to a protein mass up to ~ 50 kDa;[26] however nowadays even measurements of intact monoclonal antibodies (150 kDa) are possible.[27,28]

Still peptide analysis has several advantages over the analysis of proteins, like more efficient separation by liquid chromatography (LC), lower molecular masses resulting in fewer charge states for the desorbed and ionized peptide[29] and consequently higher sensitivity.[25] Therefore the majority of proteomics experiments use the bottom-up approach. The top down approach requires further development of MS instrumentation before it can become an “easy-to-use” technique.[24] The two approaches are visualized in Figure 8.

1.4.1 Protein digestion

Proteins are typically cleaved using proteolytic enzymes and digestion can be performed either in-solution or directly in-gel after gel-electrophoresis. For a successful and complete cleavage the tertiary structure of the protein has to be destroyed, to make cleavage sites better accessible. Therefore disulfide bonds are reduced by using chemicals like DL-dithiothreitol (DTT) or mercaptoethanol. To prevent the disulfide bonds from reforming, free cysteines (Cys) are converted into S-(carboxamidomethyl)cysteine using iodoacetamide (IAA), to 4-pyridylethylcystein using 4-vinylpyridine or S-(carboxymethyl)cysteine using iodoacetic acid.[30] These introduced modifications have to be taken into consideration for subsequent analysis.

Many proteolytic enzymes, with different cleavage specificities and varying digestion conditions, are available for enzymatic digestion, e.g. trypsin, LysC, AspN, chymotrypsin and many more.

Trypsin is the most commonly used protease, since it is available in large quantities and not very expensive.[24] It cleaves peptide bonds C-terminal of Lys and Arg, except when followed by proline (Pro). Based on an *in silico* digestion of the human Uniprot database, it was determined that trypsin generates peptides with an average length of 14 AA. Another advantage of trypsin is that the generated peptides usually contain at least two positive charges: One at the N-terminus,

and one at the C-terminal AA (Arg or Lys). Thus tryptic peptides are very well suited for MS analyses and CID-based LC-MS/MS in particular.[31]

Immobilization of trypsin is also a commonly used approach. Solubilized trypsin without carrier material should be added in a very low protein-to-protease ratio for digestion to avoid interferences of autoproteolysis peptides from trypsin during analysis.[32] Due to immobilization of trypsin it is possible to add a much higher concentration of trypsin, since immobilization reduces autoproteolysis drastically. Therefore digestion can be carried out much faster and digestion times can be reduced compared to solubilized trypsin.[33]

Multiple enzyme digestion, which is the use of more than one protease for digestion, has lately become a commonly used technique. It is suggested that this is the only way to achieve 100% sequence coverage (SC). Combinations with trypsin are mostly used because of its above mentioned benefits. Yet, tightly folded proteins are usually resistant to trypsin digestion. Also trypsin activity is lowered by many reagents. These shortcomings can be compensated by LysC.[34] LysC cleaves peptidic bonds C-terminal of Lys, thus larger tryptic peptides are formed that are further digested by trypsin if an Arg is present. Digestion becomes more efficient but data analysis is not complicated. [24]

It was shown that digestion with sole trypsin leaves over 20% missed cleavages in a digestion of yeast protein extract; whereas most of these missed cleavages are C-terminal of Lys. Supplementing trypsin with LysC reduces these missed cleavages.[34]

In this study a bottom up approach was used. Proteins were digested in solution in a volatile ammonium hydrogen carbonate buffer. Since trypsin works best at basic pH, NH_4HCO_3 (pH 8.5) is commonly chosen for trypsin digestion. Urea and thiourea are also included in the digestion buffer, to keep proteins in solution and to prevent aggregation. Disulfide bond reduction was performed using DTT and free Cys was carboxamidomethylated using IAA.

To find out if multi enzyme digestion would increase SC, immobilized trypsin and a combination of trypsin and LysC were used for protein cleavage and results were compared.

1.4.2 Desalting methods

Before measuring samples by MS, they often need to be desalted and purified. The electrospray process (see chapter 1.6.1) is especially sensitive to buffers, salts and detergents. Ionization efficiency can be lowered, since salts and buffers cause high ion emission and thereby compete with analyte ion emission. Salts will furthermore crystallize and enclose analytes.[35]

Samples are commonly desalted by using reversed phase material. More information on reversed phase is given in chapter 1.5.1. C_{18} material is used for peptides and C_4 for proteins, since a longer alkyl chain means higher hydrophobicity and proteins might be irreversibly bound to C_{18} material. The samples are bound to the stationary phase in an aqueous solvent. Then the material is washed several times with an aqueous solvent to remove interfering substances. Finally peptides are eluted with an organic solvent from the stationary phase, e.g. ACN.

In this work sample desalting and purification were carried out using C_{18} ZipTips® (Milipore, Bedford, MA, USA) and C_{18} spin columns (Thermo scientific, Rockford, IL, USA). C_{18} spin columns can process a larger amount of solution and sample, while the second step using C_{18} ZipTips® ensured complete purity and prevented clogging of the column.

1.5 Liquid chromatography

High performance liquid chromatography (HPLC) is an important tool for separation, filtration and characterization of various molecules.

Instrument set-up

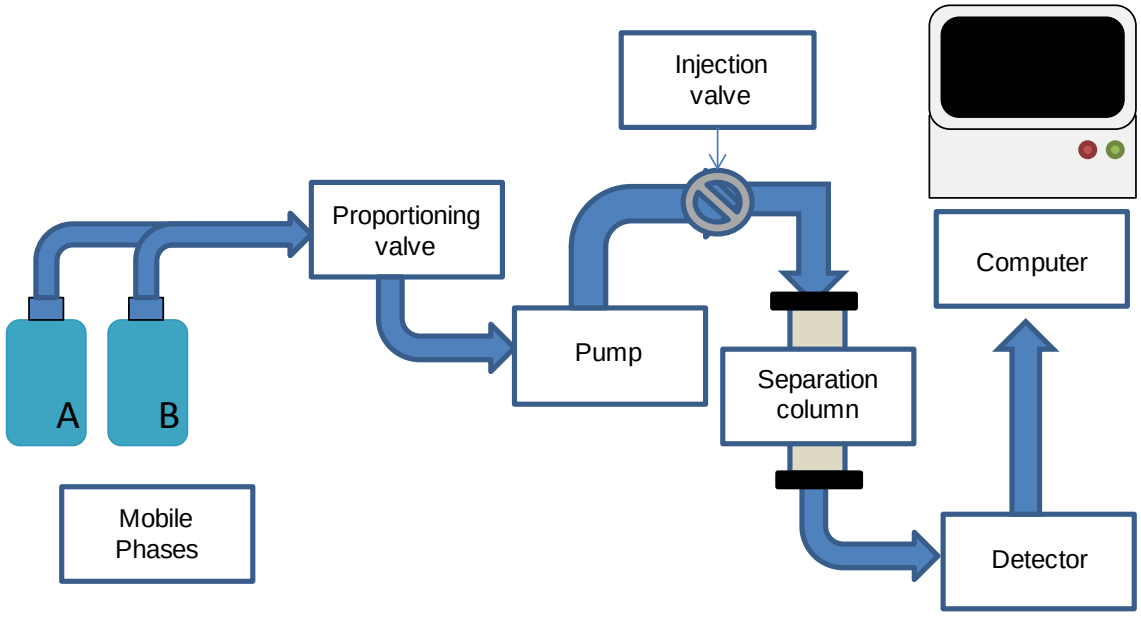


Figure 9 Basic setup of an HPLC system

The set-up of an HPLC system can be seen in Figure 9. The sample is usually injected via an auto sampler. The mixture of solvent and sample solution is forwarded to the separation column. The different substances are then analyzed by a detector and the information is sent to a

computer for data analysis. The column is operated under elevated pressure. The introduction of HPLC provided various enhancements in respect to analysis times and resolution.[36]

Separation in an HPLC system is based on the distribution of dissolved analytes between a stationary and a mobile phase. The stationary phase is a fixed solid in a separation column and the mobile phase is a liquid used for transportation of the analytes.[37]

After sample application onto the column, the different analytes interact with the stationary and mobile phase in a specific way. Analytes that have a higher affinity to the stationary phase will take more time to pass the column, than analytes that have less to no affinity to the stationary material. Analyte elution can be performed isocratic or with a gradual increase of elution solvent. After analytes have passed the column they are detected, e.g. by MS or UV spectroscopy. The result is a chromatogram that plots eluent signal intensity over time (see Figure 10). The resulting retention times (RT) are a characteristic feature for the analytes.[38]

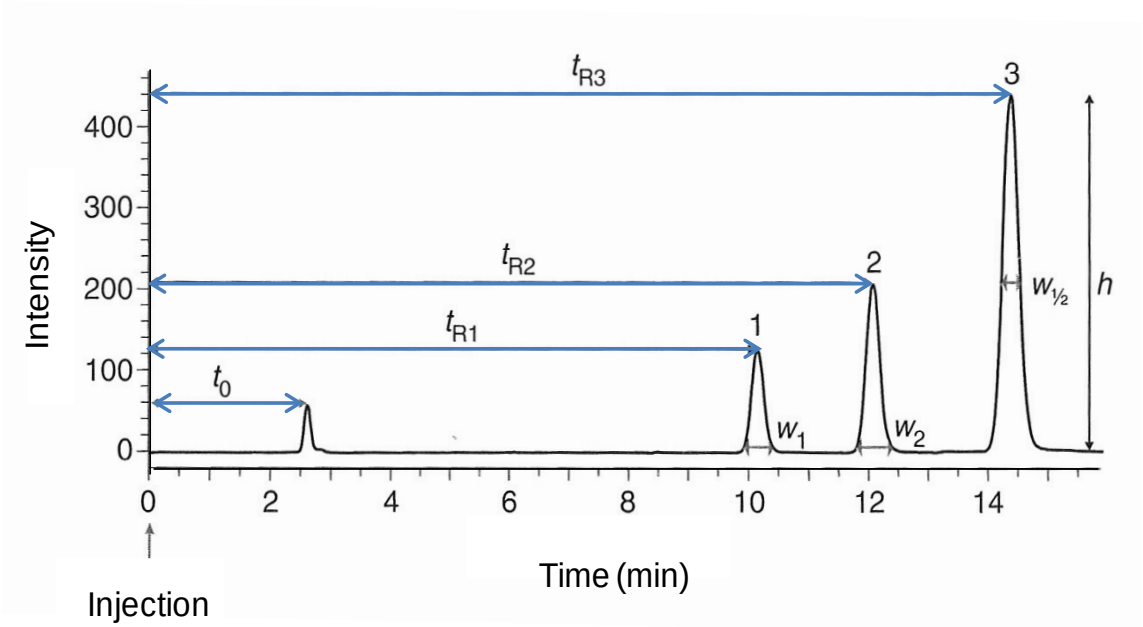


Figure 10 Scheme of a chromatogram. t_0 = RT of a non retained compound. t_R = RT of a retained compound. h = peak height, w = peak width at the baseline. $w_{1/2}$ = peak width measured at half peak height[37]

To describe a component´s retention independent from column parameters or flow rate the retention factor *k* is used, whereas t_0 is the RT of a non retained compound and t_R is the RT of a retained compound (Eq. 1.1).

$$k = \frac{t_R - t_0}{t_0} \qquad [1.1]$$

The retention factor can range from 0 (= no retention) to infinity (= irreversible adsorption).

The analytes must have different retention factors to be appropriately separated. Selectivity α is the difference in retention of one substance relative to another. α = 1 means that the substances cannot be separated (Eq. 1.2).

$$\alpha = \frac{k_2}{k_1} \qquad [1.2]$$

To evaluate the separation quality, the peak width needs also to be considered. Resolution R_S describes the relation between peak distance and peak width (Eq.1.3).

$$R_S = \frac{t_{R2} - t_{R1}}{\left(\frac{1}{2}\right) \cdot (w_1 + w_2)} \qquad [1.3]$$

t_{R1} and t_{R2} are the RTs of two peaks and w_1 and w_2 are their peak widths. R_S = 0 means no separation, R_S = 1 means partial separation and R_S > 1.5 means that the peaks are baseline separated.[37]

Various HPLC-methods can be applied for protein and peptide analysis. For ideal separation the separation method should be chosen according to the analyte´s properties. Possible separation methods are displayed in Table 2.

Table 2 Chromatography separation methods[39]

Separation method	Analyte characteristics
Size exclusion chromatography	Size, hydrodynamic volume
Reversed-phase chromatography	Hydrophobicity
Normal-phase chromatography	Polarity
Hydrophilic interaction chromatography	Hydrophilicity
Aqueous-normal-phase chromatography	Hydrophilicity
Hydrophobic interaction chromatography	Hydrophobicity
Anion-exchange chromatography	Negative charge
Cation-exchange chromatography	Positive charge
Affinity chromatography	Biospecificity
Immobilized-metal affinity chromatography	Complexation

Since reversed phase chromatography (RPC) was used in this study, it will be explained in more detail.

1.5.1 Reversed phase chromatography

The most commonly used method for peptide and protein analysis is RPC. The name “reversed phase” stems from the fact that polarity of the stationary and mobile phase is contrary to those used in normal phase chromatography. The RPC column contains a stationary phase, which usually consists of solid, sometimes porous silica material with immobilized unpolar ligands, e.g. C_4- or C_{18}-ligands. The analytes are loaded onto the column in an aqueous, polar solvent and eluted with a mobile phase containing organic solvent, e.g. ACN. Elution power for organic solvents in RPC is described by the elutropic series (Figure 11).

Water < Methanol < ACN < *n*-Propanol < Tetrahydrofuran

Figure 11 Elutropic series for organic solvents

The analytes are thereby separated according to their relative hydrophobicity. AA side chains can be polar (hydrophilic) or apolar (hydrophobic). AA composition of a peptide thereby influences RTs. Very polar peptides will pass straight through the column without retention.[39]

In this study peptide separation was performed on C_{18} columns and ACN was used as organic solvent. Formic acid (FA) was added to receive an acidic pH value. For better separation of the peptides ACN concentration of the mobile phase was increased gradiently. Since the samples were already desalted by binding to C_{18} material and eluted with 50% ACN in ultra high quality (UHQ) water / 0.05% FA, peptide elution was performed with a gradient up to 50% ACN / 0.05% FA.

1.5.2 Nano-liquid chromatography

The term nano-liquid-chromatography (nLC) refers to the low flow rates used for this application. Flow rates for nLC are usually in the nl per min range compared to µl or even ml per min for capillary and standard HPLC, respectively. The technique uses columns with a very small internal diameter (usually 75 µm). The low flow rates needed for nLC can be achieved by equipping the instrument with a flow splitter or special pumps.

The biggest advantage of nLC is the enhancement of separation efficiency and therefore sensitivity due to the smaller column. This means that sample amount can be reduced and also very limited samples can be measured.[40]

By reducing the columns internal diameter from d_1 to d_2 sensitivity is enhanced by factor f (Eq.1.4).

$$f \sim \frac{{d_1}^2}{{d_2}^2} \qquad [1.4]$$

For this to be true however, all other column parameters, like flow rate and column length, have to be kept constant and equal amounts of analyte have to be measured. Downscaling from a LC column of 4.6 mm internal diameter to 50 µm internal diameter would then lead to an increase in sensitivity by a factor 8500.[41]

At this point it has to be mentioned that the high sensitivity of nLC-based separations is also caused by the nano electrospray ionization (nESI). For further information see chapter 1.6.1.

1.5.3 Ultra performance liquid chromatography

The need for analytical laboratories to increase sample throughput has led to considerable interest for a fast LC technology, while keeping separation efficiency.[42]

In 2004 the company Waters introduced “Ultra Performance Liquid Chromatography” (UPLC) technology. It is a variation of HPLC that uses columns of standard dimensions (diameter and length), yet with particle sizes less than 2 µm (usually 1.7 µm). The small particle size leads to improved resolution and sensitivity and to shorter analysis times.[42] The downside of small particle packed columns is that such UPLC columns generate very high back-pressure. Therefore special instrumentation for UPLC is needed.[43]

“UPLC” is actually a Waters Corporation trademark, but it is also used as a name for the technique in general and is today also used as abbreviation for “Ultra High Pressure Liquid Chromatography”.

1.6 Mass spectrometry

MS is an analysis method to determine the *mass-to-charge-ratio* (m/z) of ions in a high vacuum. The basic set-up of a mass spectrometer is displayed in Figure 12.[44]

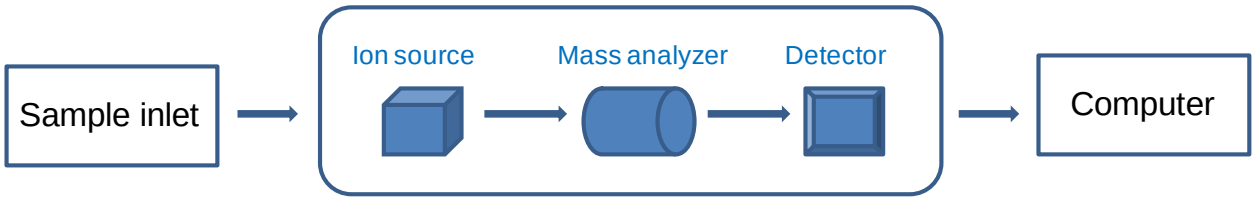


Figure 12 Schematic display of the basic components of a mass spectrometer[44]

The basic components of a mass spectrometer are ion source, mass analyzer and detector. The ion source generates ionized analytes, the mass analyzer separates the ions according to their m/z value and the detector registers the number of ions at each m/z value.[45] Application of high-vacuum in a mass spectrometer is needed to prevent collisions of analyte ions with other gas molecules.[46]

Definitions

Mass can be defined in three ways:

- *Monoisotopic mass* is calculated from the exact mass of the most common isotope of an element.
- *Average mass* is the mass calculated from the average atomic mass of each element by taking all possible isotopes into consideration.
- *Nominal mass* is calculated using the integer atomic masses of the most frequent isotope of the contained elements.
-

Mass resolution R is an important characteristic for mass analyzers. It describes a mass analyzer´s ability to detect ions even with small mass differences separately from each other. It is thus defined as the quotient of one ion mass *m* and the mass difference *Δm*, to the next separately detectable ion (Eq. 1.5).

$$R = \frac{m}{\Delta m} = \frac{m_1}{(m_2 - m_1)} \qquad [1.5]$$

For this definition it is also important to declare at which point two peaks are distinguished as separated. When quadrupoles are used for ion separation, two peaks are considered to be resolved, if the valley between them is equal to 50% of the weaker peak intensity. For instruments with a high resolution, like ion cyclotron resonance (ICR) instruments, two peaks are considered separated, if the valley is at 10% of the weaker peak intensity (Figure 13 A).[47,48] Resolution can also be determined for an isolated peak. In this case *Δm* is defined as the *full width at half maximum* (FWHM), which is the peak width at 50% total peak intensity (Figure 13 B).[49]

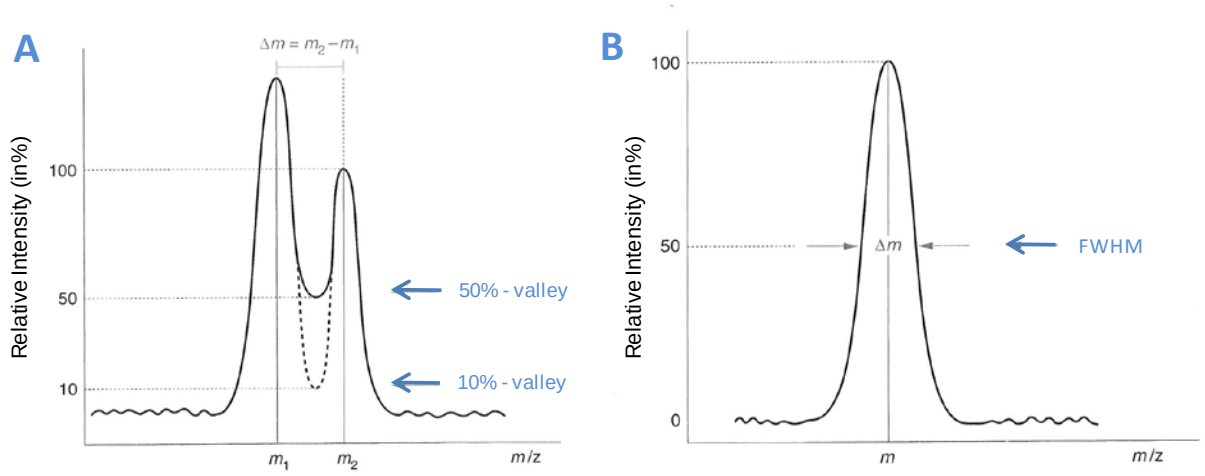


Figure 13 Mass resolution definition for mass spectrometers: (A) 10% and 50% valley method. (B) Full width at half maximum method.[49]

Another important parameter to describe a mass analyzer´s quality is *mass accuracy*. It describes the error for a measured m/z, which means by how much the measured mass differs from the correct mass value. Mass accuracy can be given either as an absolute value or as a relative value in ppm.[50]

The *signal-to-noise-ratio (S/N)* quantifies the ratio of a signals intensity relative to noise. It describes a signals quality by giving it a quantitative measure, describing the uncertainty of a measurement.[51]

The *detection limit* is defined as the lowest amount of analyte needed to produce a signal that can be clearly distinguished from the background noise. It is often confused with *sensitivity*,

which describes an analytical system´s overall response for a certain analyte under well-defined conditions of operation.[52]

Ionization, mass analysis and detection

The two most commonly used techniques for analyte ionization are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Both methods are so called “soft-ionization”-methods, which means that the analytes are transferred into the gas phase without excessive fragmentation.[53] ESI is used for analytes in solution and is therefore commonly used in combination with liquid based separation tools, like chromatography, while MALDI ionizes analytes from a crystalline matrix via laser pulses.[45]

Quadrupole (Q), time-of-flight (TOF) analyzers, quadrupole and linear ion traps (QIT, LIT), ioncyclotrons (ICR) and orbitraps (OT) are frequently used mass analyzers.[49]

For ion detection faraday cups, secondary electron multiplier and multichannel plates are commonly used.[54]

In this study LC-MS/MS was performed. Therefore ESI and the used mass analyzers (ion trap and triple quadrupole) are explained in more detail in the following chapters.

1.6.1 Electrospray ionization

ESI is a soft ionization technique used for MS. The term electrospray describes dispersion of a liquid into many small charged droplets in an electrostatic field. ESI leads to desolvation, which means that ions are transferred from solution to the gas phase at atmospheric pressure.[35]

The idea of applying electrospray on a liquid containing analytes to make them applicable for MS was first described by Dole et al. in 1968.[55] In the early 1980s Fenn and his group[56] developed the idea further and demonstrated the use of ESI as a MS technique for large molecules. Mann introduced nESI in 1996, which is today’s most sensitive ESI mode.[57]

Instrument set-up

The basic setup of an ESI ion source consists of a spraying needle, a potential gradient and a drying gas. Clogging of capillaries is one of the most recurring problems in ESI, especially if involatile substances are present in the sample. In the beginning the ESI needle tip was in line with the entrance to the mass analyzer, introducing these non-volatiles into the high vacuum of the analyzer. To reduce this unwanted introduction of interfering substances orthogonal spraying

was introduced. Another advantage of orthogonal spraying is that only small and highly charged droplets are directed towards mass analysis, since large, less charged droplets cannot be sufficiently attracted by the electric field at a higher angle.[58,59]

The ionization process

The process is roughly subdivided into 4 basic steps:

- Formation of small droplets
- Vaporization of solvent solution leading to an increase in charge density on the droplet surface
- Breakup of the droplets into micro droplets due to Coulomb explosions
- Desolvation of analytes during transfer to the mass analyzer

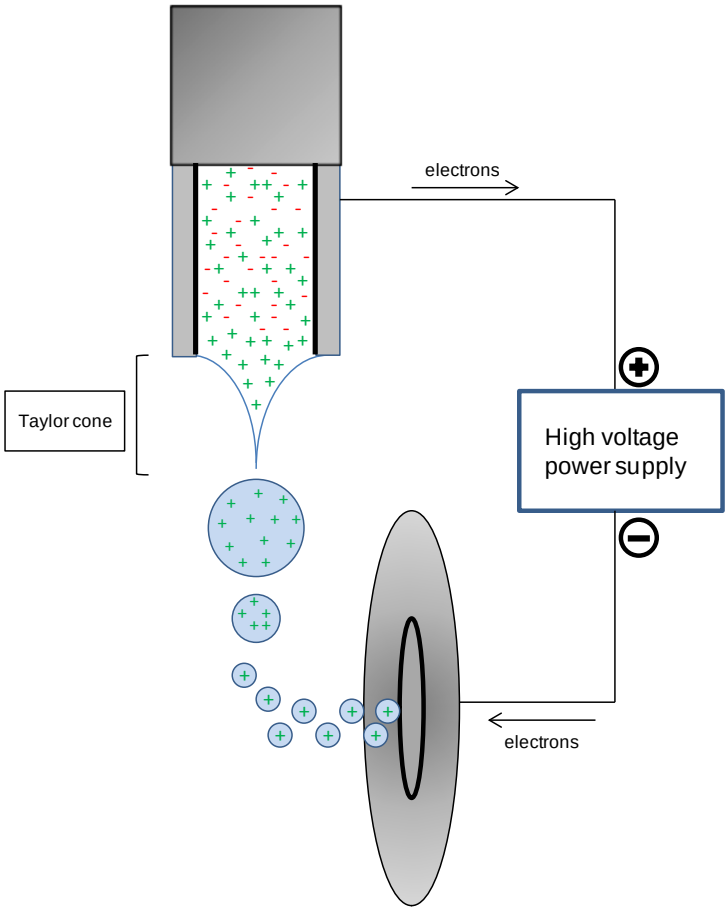


Figure 14 Display of the electro spray ionization process for positively charged ions

Figure 14 shows the ESI process for positively charged analytes. The solvent and analytes are continuously delivered to the tip of the spray capillary at atmospheric pressure. A high voltage

electrostatic field is applied between the capillary tip and the entrance (orifice, transfer capillary) into the high vacuum region of the mass spectrometer. Thereby the positively charged ions are pulled to the liquid's surface, while the negatively charged ions are pushed in the other direction. The positive ions are then pulled further to the cathode. This leads to the formation of the "Taylor cone", a liquid cone that forms due to counteraction of electrostatic field and surface tension. In short distance from the anode, droplets (containing many positive charges) are formed. These droplets are close to the Rayleigh-limit, which is defined by the repellent Coulomb energy of same charges and the solvent´s surface tension. A heated, inert drying gas, usually nitrogen, is introduced into the spray chamber to evaporate the solvent from the droplets. By evaporation of the solvent the radius of the droplets shrinks and the Rayleigh-limit is exceeded. After this the droplets explode and form many small daughter droplets that contain, after multiple of such steps, only one analyte molecule, due to rejection of same charged ions (Coulomb explosion).[35]

Two suggestive models exist to explain the formation of free gas-phase ions. The *charged-residue model* suggests that a series of Coulomb explosions ultimately leads to the formation of droplets with a radius of about 1 nm that only contain one analyte molecule. Desolvation occurs due to collisions with curtain gas. The *ion evaporation model* suggests that ions are emitted into the gas phase directly from droplets that still contain many analytes.[35]

nESI is also very commonly used. While in the beginning of nESI needles were filled with the sample solution, today nLCs can be directly coupled to MS. The key feature of nLC-nESI is to improve ionization efficiency by introducing a very small flow rate compared to LC-ESI (nl/min compared to ml/min). This leads to emission of charged droplets from an extremely small diameter from the Taylor cone and therefore improves sensitivity.[40] Downscaling from a standard ESI needle (~0.1 mm internal diameter) is achieved by replacing the spray needle with a borosilicate glass capillary to which a fine tip is pulled.[60] The capillary tip has a narrow exit of only 1 - 4 µm diameter. Thus a stable electrospray can be provided at flow rates of 20-50 nl/min.[57]

Typical solvents for the analysis of positively charged analytes (*positive ion mode*) are mixtures of polar and organic solvents with the addition of aqueous acids or bases, e.g. FA, acetic acid or ammonia or volatile buffers, e.g. ammonium formiate, acetate or carbonate. By coupling HPLC to MS, substances are separated and isolated before analysis and ions can be detected, that would otherwise be suppressed by more abundant ions.

Involatile salts and detergents disturb the electrospray process and should therefore be removed as part of sample preparation (See chapter 1.4.2).[35]

Ions derived from the ESI process are usually multiply charged and not fragmented. In positive ion mode charging normally occurs via protonation, however also sodium or ammonium adduct ions are very often observed.[60]

1.6.2 Quadrupole Ion Trap mass spectrometry

A QIT is a mass analyzer that can perform several important functions. The biggest advantage of the QIT is mass accumulation. Ions are collected in the trap, where selective masses can be isolated. These ions are then excited for CID.[61]

Instrument set-up

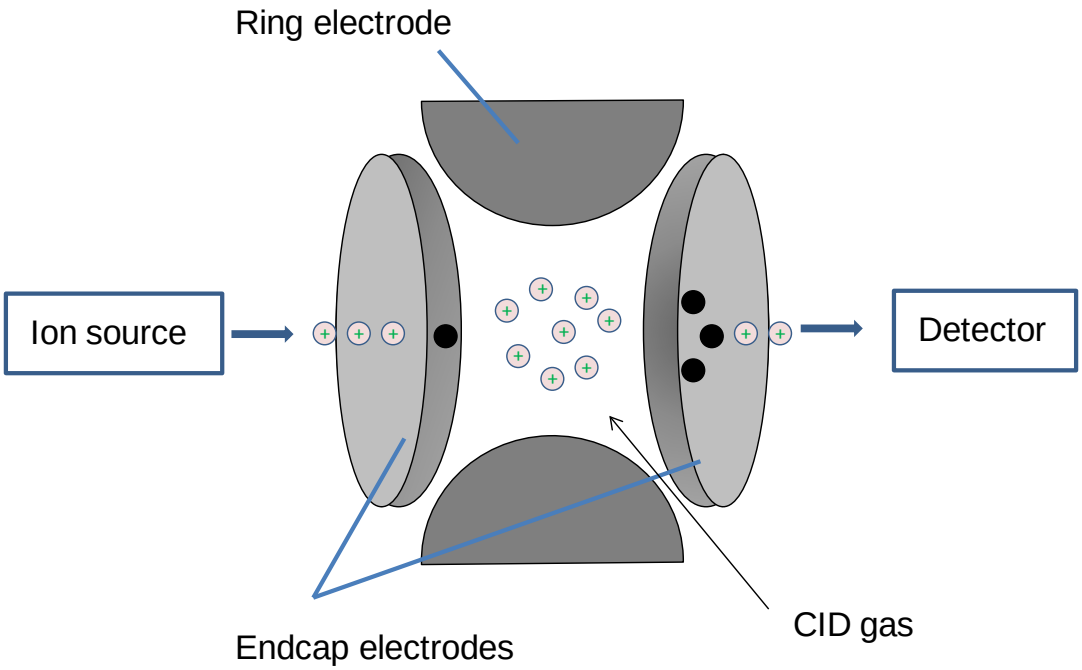


Figure 15 Scheme of a 3D quadrupole ion trap (3D QIT) for mass analysis

Ion traps exist in linear[62] as well as three dimensional shape.[63] In this study the 3D QIT – a HCT^{plus} from Bruker Daltonics (Billerica, MA, USA) was used. Therefore it will be explained in more detail.

A three dimensional electric-field (3D) QIT consist of a ring electrode and two endcap electrodes (see Figure 15). The endcap electrodes have holes in their center to allow the ions to pass in and out of the trap. A high voltage RF potential is applied to the ring electrode, while the endcap

electrodes are held at ground. This oscillating potential difference leads to the formation of a quadrupolar field. The quadrupolar field generates a potential well, where ions are stabilized. Ions of a particular m/z range are trapped inside the field, depending on the level of RF voltage.[61]

Ion trapping

Since the ions are accelerated from the ion source into the QIT, they arrive in the trap at a certain speed. To prevent the ions from simply passing through the QIT, a collision gas (usually helium) has to be present in the trap. The ion´s speed is reduced due to collision with the helium atoms, and trapping becomes more efficient. Typical accumulation times for ions in the trap for MS experiments range from 0.1 to 10 ms. After this the QIT is usually "blocked" to prevent "overfilling" of the trap. If too many ions enter the trap, it would result in too many ion collisions and subsequently imprecise m/z ratio measurements.[49]

The Mathieu´s stability diagram describes the range of ions with different masses that can be trapped at the same time. The stability diagram is a two dimensional plot that demonstrates the potentials, under which the ions are stable or unstable in the field. The axial direction is the z-axis, the direction of injection and ejection. Ion movement in the xy-axis is described by the Mathieu equation.

$$\frac{d^2 \cdot x}{d(\pi \cdot f \cdot t)^2} + [a + 2q \cdot \cos(2\pi \cdot f \cdot t)] \cdot x \qquad [1.6]$$

$$\frac{d^2 \cdot y}{d(\pi \cdot f \cdot t)^2} + [a + 2q \cdot \cos(2\pi \cdot f \cdot t)] \cdot y \qquad [1.7]$$

The parameters *a* and *q*

$$a = \frac{2z \cdot e \cdot U}{m \cdot (\pi \cdot f \cdot r)^2} \quad \text{and} \quad q = \frac{z \cdot e \cdot V}{m \cdot (\pi \cdot f \cdot r)^2} \qquad [1.8]$$

describe the relation between ion mass *m* with *z* elemental charge *e* and the characteristics of the instrument (Eq.1.8). Instrument parameters are *r* (the equatorial radius of the trap) and the

electric field, consisting of the direct current *U* and the amplitude of the radio frequency voltage *V* with the radio frequency *f.* Therefore stable oscillations on x and y axis is possible for ions with a defined m/z ratio at certain values for *a* and *q.*[64]

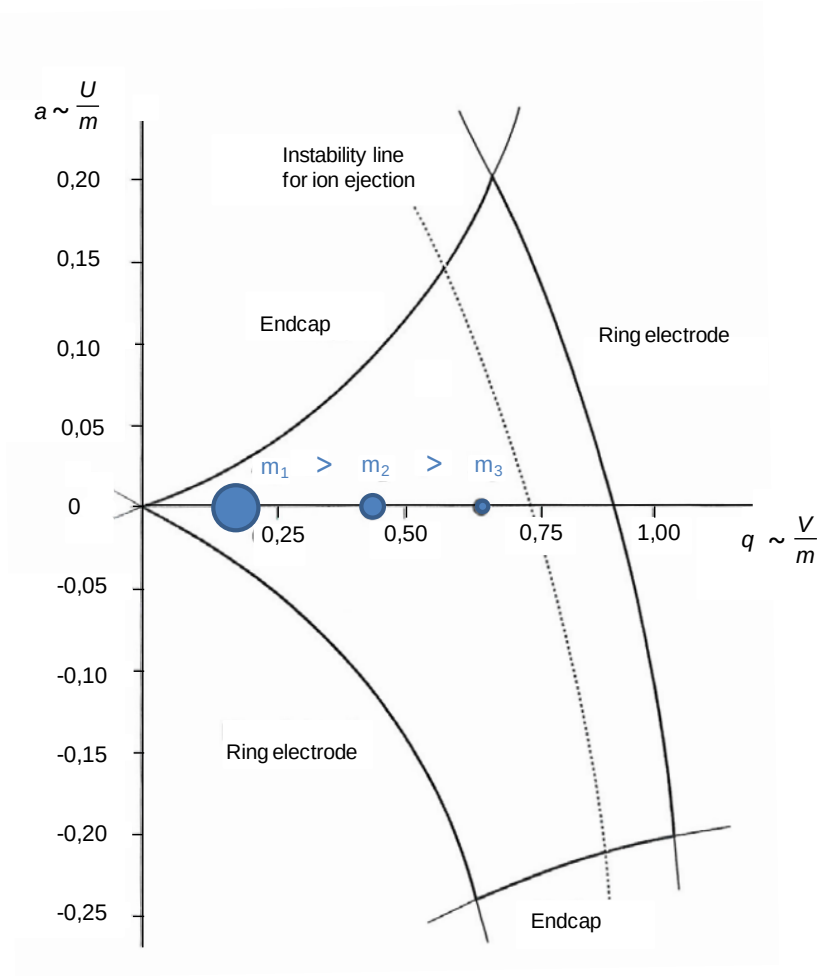


Figure 16 Mathieu stability diagram for 3D QIT[49]

From the equation of the parameters *a* and *q* results:

$$\frac{a}{q} = \frac{2z \cdot e \cdot U}{m \cdot (\pi \cdot f \cdot r)^2} \cdot \frac{m \cdot (\pi \cdot f \cdot r)^2}{z \cdot e \cdot V} = \frac{2U}{V} \qquad [1.9]$$

Solving the Mathieu equation for every a/q combination results in the Mathieu stability diagram (see Figure 16). Every point of this diagram represents ions of a certain m/z value for given values of *r*, *U, V*, and *f.*[49]

As shown in the stability diagram, the broadest mass range for ion trapping occurs when no direct current is applied (*a = 0*). This is why QITs are usually operated under radio frequency voltage only.[49]

The principal method for measuring m/z is to "point the potential well in a particular direction" by coupling the quadrupolar field of the ring electrode to a dipolar field at the endcaps, so that ions leave the trap according to their increasing m/z values.[61] Resonant animation empowers the ions extremely fast to increased oscillation, which leads to ion instability that is used for ion ejection within the real stability range.[49]

Tandem MS for QIT

A QIT is a *tandem-in-time* instrument. This means that precursor ion selection, fragmentation and acquisition of fragment ion spectra are performed in the same space. This can be utilized to not only perform MS^2, but even more fragmentation steps.[65] Latter is no longer called tandem MS, but multi-stage MS (MS^n).

Applying an alternating voltage to the end caps, which is specific to the isolated m/z value, excites an isolated ion leading to its fragmentation. The resonating ions take up energy from the dipolar field and start colliding with helium atoms that are introduced as collision gas. This causes ions to dissociate.[66] Since the voltage for excitation is specific for an ion, product ions are not accelerated and secondary fragmentation is very limited. This leads to very "clean" MS/MS spectra.[67]

The Mathieu diagram also shows an important factor for QITs: the low mass cut-off (LMCO). LMCO means that the QIT fails to trap ions at a low m/z range, which is especially critical when CID is used.[68] For CID a *q* value is used that increases the kinetic energy of the parent ion via power absorption from a resonance excitation voltage. The downside of this is that ions with a m/z value below the LMCO cannot be trapped. Therefore product ions with m/z values below ~25‑30% of the parent ion´s m/z value do not appear in the MS/MS spectrum.[69]

Furthermore it has to be said that a QIT has a limited dynamic linear range, which is a drawback for quantification (see chapter 1.7).

1.6.3 Triple Quadrupole mass spectrometry

For the development of the quantification method in this study a LC-MS 8030plus from Shimadzu Kratos Analytical (Manchester, UK) was used. It has a triple quadrupole (QQQ) mass analyzer, which is described in this chapter.

Instrument set-up

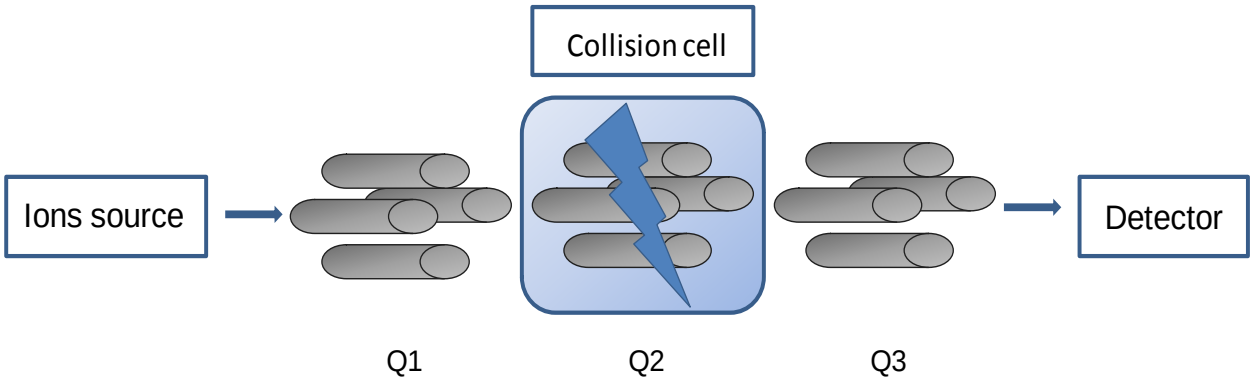


Figure 17 Scheme of the setup of a triple quadrupole mass analyzer

The setup of a QQQ can be seen in Figure 17. A Q consists of 4 parallel rod-shaped electrodes. A QQQ therefore consists of 3 Qs, which are sequentially aligned. Q1 is used to scan the ions. Q2 is used as a collision cell for ion fragmentation. It is filled with an inert collision gas. In this work argon was used for MS/MS experiments. Q3 is then used to scan the fragment ions.[49]

The operation principle in a QIT and a single Q is very similar. The two opposite rods are always synchronized and either direct or alternating current with a certain frequency *f* is applied. This creates an electric potential along the z-axis. Ions with defined m/z values are passed through the mass analyzer at specified combinations of alternating and direct currents. On the contrary to a QIT, which accumulates ions, a Q is therefore like a mass filter.[49] Due to the geometry of the Q, which differs significantly from a QIT, the Mathieu stability diagram is also different. The region where $a = 0$, is symmetric.[70] In Figure 18 only the upper part of the diagram is displayed.

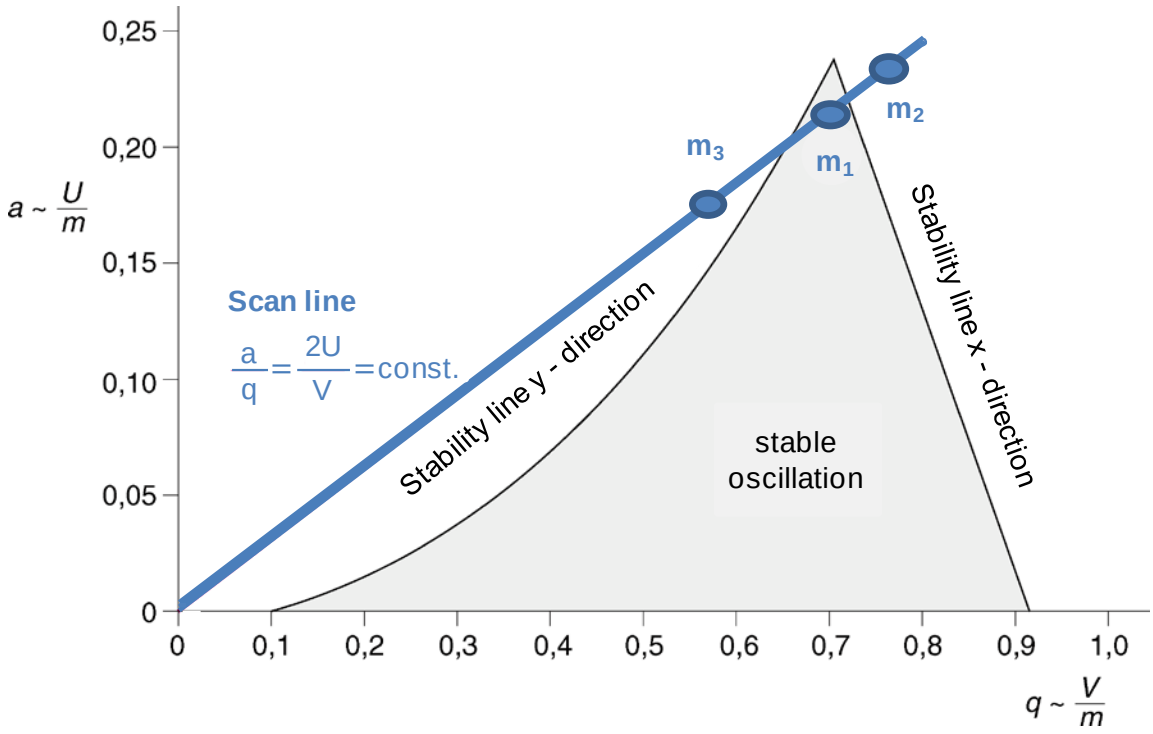


Figure 18 Mathieu stability diagram for a 2D quadrupolar field in x and y direction[49]

Tandem MS for QQQ

CID in a QQQ differs significantly from a QIT. In a QQQ all ions are subjected to collisional excitation during their passage through Q2. This leads to frequent secondary fragmentation of fragment ions.[67] Therefore the collision energy (CE) has to be well adapted.

b-ions of tryptic peptides give usually rather weak signals compared to y-ion signals, when CID is performed in a QQQ (b/y-ion explanation see chapter 1.6.5 and Figure 22). It has been suggested that this happens due to secondary fragmentation of b-type ions, which reduces peak intensities for higher m/z values and increases peak intensities for lower m/z value. y-type ions turn out to be more stable through proton sequestration by the C-terminal basic residue side chain.[71]

Different scan modes for MS/MS can be applied in a QQQ. Product ion scan, precursor ion scan, neutral loss scan and MRM are explained in more detail for peptide analysis. The different scan modes are displayed in Figure 19.

Product ion scan

For a product ion scan the Q1 is fixed to filter ions of a certain m/z value. These so called “precursor ions” are then fragmented in Q2. All resulting fragment ions (“product ions”) are then scanned in the Q3. Product ion scan is used for identification and even for quantification of a single component in a mixture of analytes.[72]

Precursor ion scan

If a precursor ion scan is performed, all ions that arrive at the Q1 are scanned and CID is executed in the Q2. Q3 is then fixed to only let product ions of a certain m/z value pass through. Ions are only detected, if a precursor ion in Q1 produces the according product ion. The mass spectrum shows all ions that are scanned in Q1, when the desired product ion reaches the detector. Precursor ion scan is commonly used to identify a peptide modification due to specific product ions, produced by the modification.[72]

Neutral loss scan

For a neutral loss scan all ions are scanned in the Q1, followed by a fragmentation step in Q2. Q3 is not fixed to a specified mass, but constant mass differences to Q1 are measured. The mass spectrum shows all precursor ions that deliver a product ion with this predefined mass difference. Just like the precursor ion scan, a neutral loss scan is commonly used to identify peptide modifications, e.g. phosphorylation.[72,49]

Multiple reaction monitoring

MRM means that specified fragmentation reactions are monitored. For this scan method Q1 as well as Q3 are fixed to certain m/z values. The ions selected in the first Q only produce a signal if the corresponding fragment ion is built.[72] MRM can be used for very sensitive and specific detection of known compounds in complex samples.[49]

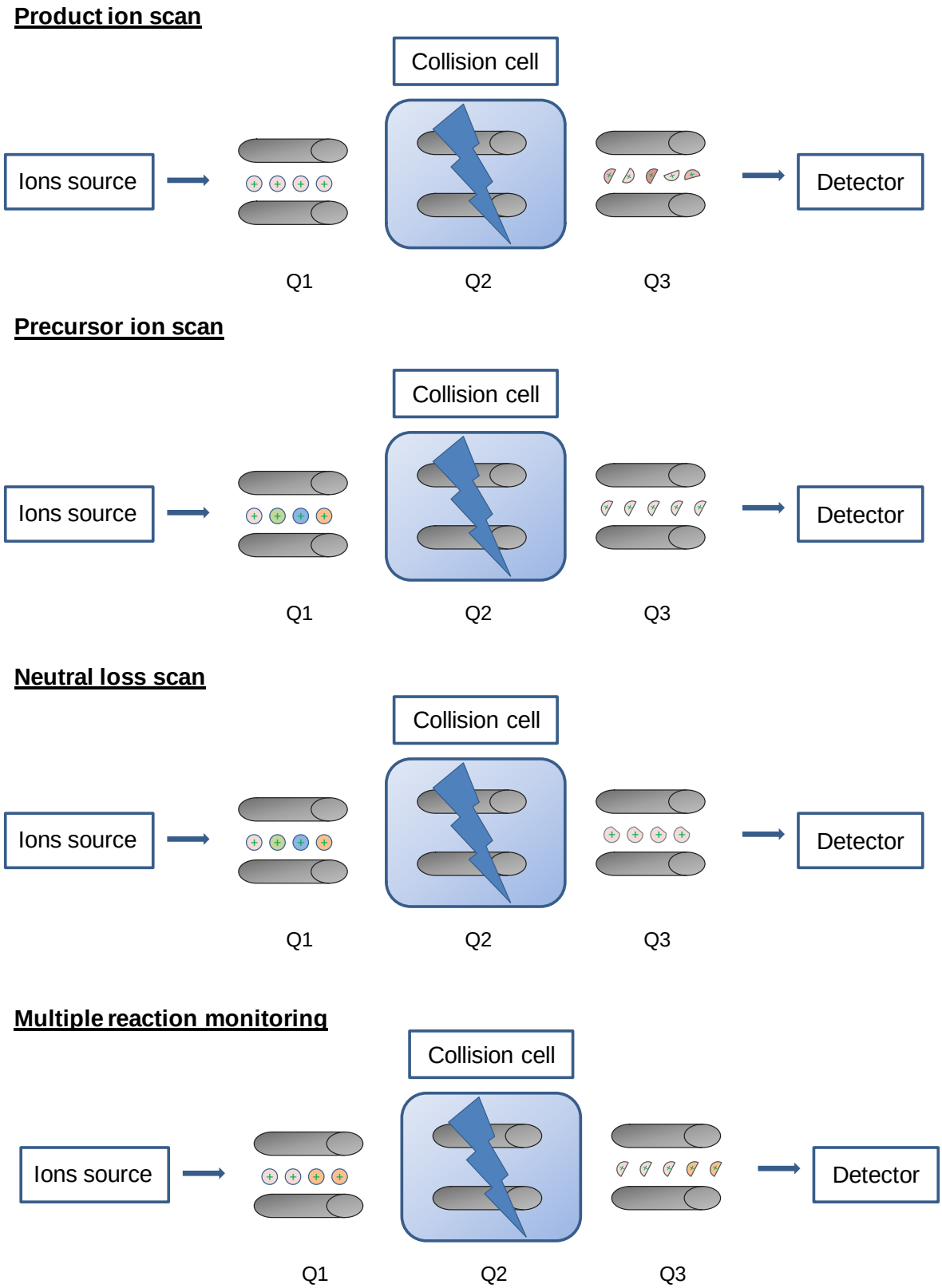


Figure 19 Principle of product ion scan, precursor ion scan, neutral loss scan and MRM for MS/MS analysis in a QQQ

1.6.4 Electron multiplier

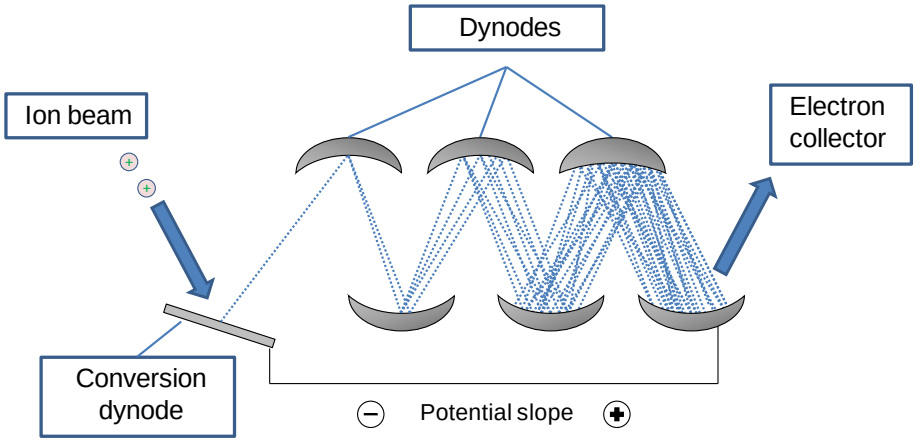


Figure 20 Principle of a discrete dynode secondary electron multiplier

An electron multiplier is commonly used as a detector for MS.[73] Ions from the mass analyzer are accelerated to a high velocity in order to enhance detection efficiency. An electrode, called a conversion dynode, is held at high potential, opposite to the charge of the detected ions. If positive ions encounter the negative conversion dynode, electrons are built as secondary particles. The electron multiplier then amplifies the electrons to form a current. The principle of a discrete dynode secondary electron multiplier (SEM) is shown in Figure 20.[74] Secondary electrons can also be produced in a continuous tube. Such detectors are called channel electron multipliers (CEM). CEMs are less expensive and also more compact than discrete dynode SEMs (Figure 21).[75] Another option would be a micro-channel plate (MCP). A MCP consists of a plate, which has multiple channels (Ø 4 – 25 µm).[74] The channels are drilled at a certain angle (~8°) to the surface.[76] After ions hit the front side of the plate, they produce secondary electrons, which trigger a cascade down the channel.[73]

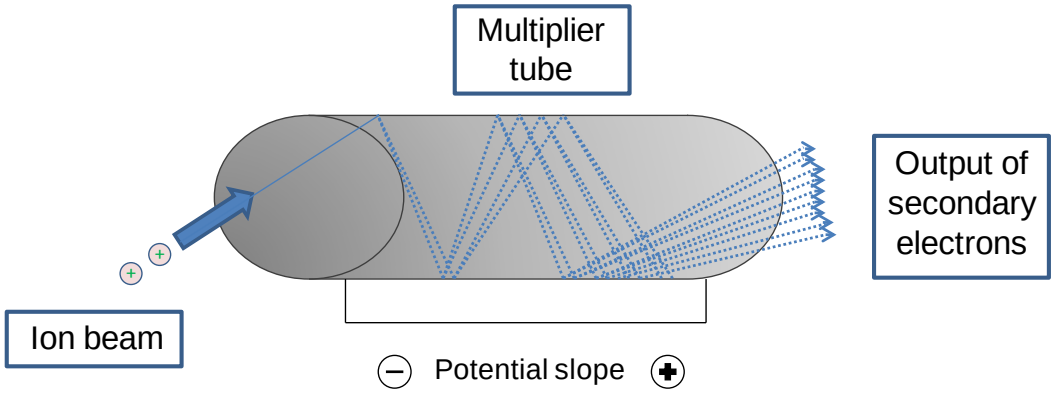


Figure 21 Principle of a CEM

1.6.5 Peptide sequencing and identification

Molecular mass is determined by the molecular formula of the included elements.[77] Different AA sequences can still result in isobaric peptides. Thus for correct protein identification not only peptide mass, but also the peptide sequence is very important.[78]

Due to their capability of MS^n and the fact that MS and MS/MS spectra are subsequently recorded, QITs are commonly used for protein identification experiments. Low-energy CID experiments usually result in fragmentation of the amide bonds and very little side chain fragmentation is observed. Therefore the spectra are easy to interpret.[78]

Roepstorff and Fohlman[79] established a nomenclature for peptide fragmentation (see Figure 22). The nomenclature was later modified by Biemann.[80,81]

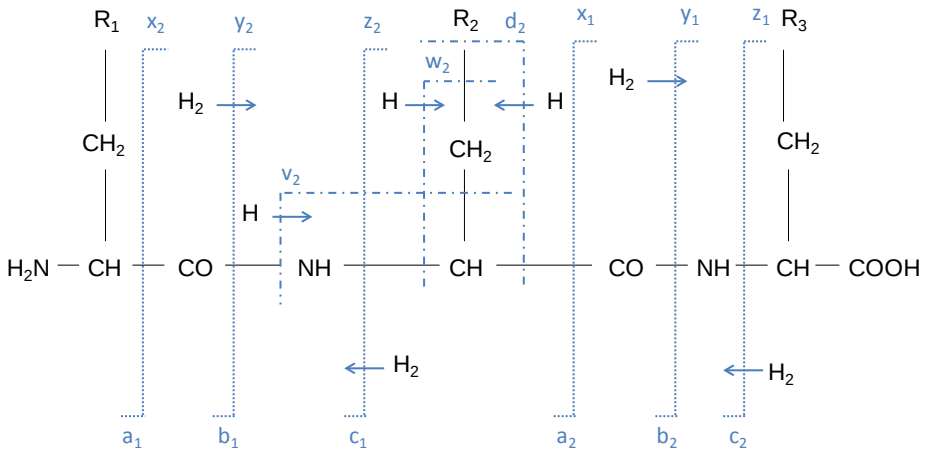


Figure 22 Nomenclature of peptide fragmentation [79,81] (adapted from Lottspeich and Engels[77])

Fragment ions that contain the intact N-terminus are called a, b and c ions. Fragment ions which contain the intact C-terminus are called x, y and z ions. The number behind the letter indicates the number of AAs contained in the fragment.[77]

The fragmentation pattern of peptides is dependent on AA composition, size of the peptide, excitation method and charge state of the ion. Under low-energy collision conditions (1-200 eV) mostly b- and y-ions are formed (high energy CID > 1 keV). The peptide sequence can be determined by MS/MS. The mass differences of consecutive fragment ions of the same type (e.g. b-ions or y-ions) give information on the AA residue. In this way even *de novo* sequencing (= AA sequence determination) is possible.[71] In Figure 23 an example for peptide sequencing by interpreting MS/MS spectra is given.

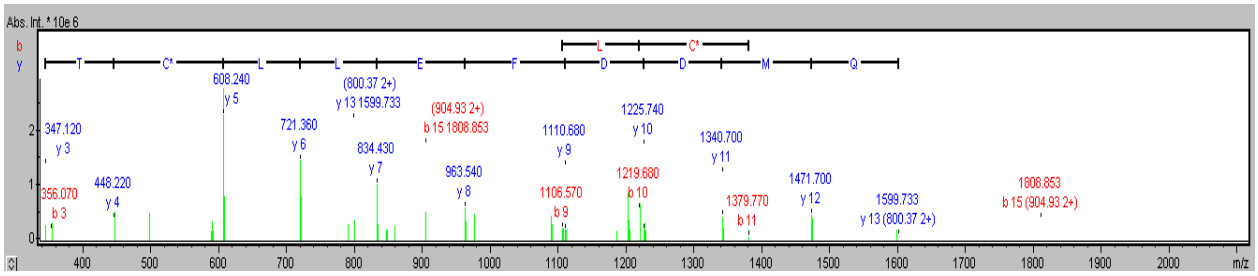


Figure 23 MS/MS spectrum of the peptide sequence K.NLQMDDFELL(cam)CTDGR.R illustrated in BioTools v3.2. b ions are displayed in red, y ions are displayed in blue.

Another approach for protein identification is to compare the obtained spectra with a database. Available databases are for example NCBInr (http://www.ncbi.nlm.nih.gov/) or UniProt.[82] The search can be performed using various search engines. The most commonly used search engines for MS/MS spectra are Mascot[83], Sequest[84] and X!Tandem[85]. For protein identification via database search the protein sequence has to be already known and available in the searched database.[86]

In a database search proteins are digested *in silico* by a program according to the inserted search parameters, i.e. enzyme type for digestion, chemical modifications of peptides and mass accuracy of measurement. A list of theoretical masses is produced that is then matched to the measured masses. By using a specified cleavage reagent for protein digestion, AAs at the N- or C-terminus can be taken into account as predefined. If trypsin is used for enzymatic cleavage the C-terminal end of the peptide is always either Arg or Lys and the N-terminal AA is one that follows Arg or Lys in the protein sequence.[78] Thereby the number of possible matches is reduced drastically. Other important parameters to narrow down the search are taxonomy, the peptide mass tolerance and the number of allowed missed cleavages. Further fixed and variable modifications can be specified. Modifications can be biologically relevant (e.g. phosphorylation), or introduced during sample preparation like carbamidomethylation or oxidation of methionine.[83]

Theoretical MS/MS fragment ions of *in silico* peptides are then compared to the actually measured ions.[86] Each comparison is ranked by a score that indicates the statistical probability of a match.[78]

1.7 Quantification

The development of protein quantification methods is a very challenging field of proteomics. While quantification can be very useful to answer biological and biomedical questions, the best suited method for a project can only be developed after considering multiple factors, such as the source of the samples, the time required and the type of available equipment.[87]

Basically there are two types of quantification methods: absolute quantification and relative quantification. Absolute quantification means, that it is possible to determine the exact amount or concentration of a sample. Relative quantification is only a comparison of results and can be used for example to determine protein up- or down-regulation. However even absolute quantification is only relative to an internal standard.[87]

MS is in principle not a quantitative method. Depending on their physicochemical characteristics, ionization efficiencies between different analyte molecules can be very different. Therefore it is not possible to conclude on the concentration of different analytes in a sample by simply comparing signal intensities. It is however possible to compare chemically identical analytes in other, but similar, samples.[88]

At the moment there is no MS quantification method that can be recommended as the “best” method. The method best suited for the experiment has to be chosen according to the external preconditions.[87]

As already mentioned before, the dynamic linear range for quantification in a QIT is limited. Accumulation of ions within the trap is limited by the geometric and electrical properties of the trap.[89] If too many ions are accumulated in the rather small space inside trap, the maximum charge density would be exceeded and ions are repulsed, leading to non linear results in respect to ion number.[90]

Today, QQQ instruments are preferred for quantification due to their high sensitivity when using MRM assays that allow for a wide linear dynamic range for multiple analytes within one sample.

For this study a LC-MS/MS quantification method was developed on a QQQ instrument. The decision was based on the available equipment at Romer Labs. A relative quantification method was established.

2 Experimental

2.1 Materials

2.1.1 Chemicals and Equipment

- Chemicals:

Amresco (Solon, OH, USA):

- **Urea,** High purity grade

  Prod# 0568-1KG, LOT# 1273C464

Fluka (Buchs, Switzerland):

- **Acetonitrile**, LC-MS-CHROMASOLV®

  Prod# 34967-2,5l

- **Ammonium hydrogen carbonate**, ≥99.5%

  Prod# 09830-100G, LOT# 446338/1

- **Conalbumin from hen egg,** ≥89%

  Prod# 27695-500mg, LOT# 296661/1

Merck KGaA (Darmstadt, Germany):

- **Formic acid**, 98-100% pro analysi

  Prod# 1.00264.1000-1L, LOT# K37957664 744

Milipore (Bedford, MA, USA):

- **Ultra high quality water (UHQ)**, was obtained by using a Simplicity system with 18.2 MΩ × cm resistivity at 25 °C

Promega (Madison, WI, USA):

- **Trypsin/LysC mix**, Mass spec grade

  Prod# V5071, LOT# 0000082174

  Prod# V5072, LOT# 0000112174

  Prod# V5072, LOT# 0000121726

Sigma-Aldrich (St.Lois, MO, USA):

- **Conalbumin from chicken egg white,** substantially iron free

  Prod# C0755, LOT# 107K7022

- **DL-Dithiothreitol,** Bio Ultra, ≥ 99.5%

  Prod# 43815-5G, LOT# BCBD7009V

- **Iodoacetamide**, Bio Ultra

  Prod# I1149-25G, LOT# SLBD7510V

- **KRTLRR trifluoro acetate salt,** 98%

  Prod# L-9905, LOT# 44H58054

- **N-Hydroxysuccinimide**

  Prod# H-7377, LOT# 66H36211

- **N,N´- Dicyclohexylcarbodiimid,** 99%

  Prod# D8000-2, LOT# S15587-104

- **Thiourea**, Minimum 99.0%

  Prod# T7875-500G, LOT# 033K0123

Thermo Scientific (Rockford, IL, USA):

- **Immobilized TPCK Trypsin,** 2 ml of settled gel supplied as a 50% slurry containing glycerol and 0.05% sodium azide

  Prod# 20230, LOT# PD200057

- Equipment:

Milipore (Bedford, MA, USA):

- **ZipTip® C_{18} Pipette Tips**, Tip size: P10

  Prod# ZTC18S960, LOT# R3NA23232

Thermo Scientific (Rockford, IL, USA):

- **Pierce® C-18 Spin columns**

  Prod# 89870

VWR (Fontenay-sous-Bois, France):

- **Centrifugal filter**, modified PES, 10K, 500 µl

  Prod# 516-0229, LOT# FZ1523

2.1.2 Instrumentation

- **UltiMate® 3000 Nano LC system** - Pump, flow manager and autosampler, Nano LC System, Dionex (Sunnyvale, CA, USA)
  - Column: **Acclaim PepMap®**, C_{18}, Ø 75 µm, length: 150 mm, particle size: 3 µm, pore size: 100 Å, Thermo Scientific (Rockford, IL, USA)
- **HCT^{plus}** – Ion Trap mass spectrometer, Bruker Daltonics (Billerica, MA, USA)
- **Nexera system LC-30AD** - liquid chromatograph, SIL-30AC auto sampler, CTO-20AC column oven, CBM-20A communication module, Shimadzu Kratos Analytical (Manchester, UK)
  - Column: **Acquity UPLC® BEH300**, C_{18}, Ø 2.1 mm, length: 100 mm, particle size: 1.7 µm, pore size: 300 Å, Waters (Manchester, UK)
- **LC-MS 8030plus** - liquid chromatography mass spectrometer, Shimadzu Kratos Analytical (Manchester, UK)
- **pH meter MP220**, Mettler Toledo (Schwarzenbach, Switzerland)
- **Micro centrifuge Mini Star silverline**, VWR (Radnor, PA, USA)
- **Ultrasonic cleaner**, VWR (Radnor, PA, USA)
- **Analog vortex mixer**, VWR (Radnor, PA, USA)
- **Thermomixer comfort**, Eppendorf AG (Hamburg, Germany)
- **Nano photometer,** Implen (Germany)
- **Vacuum centrifuge** UNIVAPO 100H with UNICRYO MC2L -60°C, Uni Equip (Planegg, Germany)
- **Sigma 1-14 Microcentrifuge**, Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany)

2.2 Conjugate synthesis

2.2.1 Samples provided by Romer Labs

For detailed information about conjugate synthesis see master thesis by C. Stephan.[91]

Protein conjugates were synthesized at Romer Labs and provided by Barbara Cvak. Therefore reaction conditions are described only briefly. Modification parameters are listed in Table 3 . The varying parameters for modification were coupling ratio (cr) and activation time. The cr describes the molar ratio between the CONs and ZEN-CMO. Therefore a cr of 1:50 means 50 mol ZEN-CMO per 1 mol protein were added. 8 samples with a concentration of 1 mg/ml in 0.01 M PBS buffer were provided. Samples were stored at 4°C.

Table 3 List of conjugate samples provided by Romer Labs. cr and activation time were varied in the syntheses

Sample name	cr	Activation time
A1	1:10	1 hour
A2	1:25	1 hour
A3	1:50	1 hour
A4	1:75	1 hour
B1	1:05	over night
B2	1:10	over night
B3	1:25	over night
B4	1:50	over night

2.2.2 Protein modification

In the course of this thesis sample A3 was synthesized one more time (cr = 1:50, activation time = 1 hour) to check for reproducibility.

Working solutions:

Table 4 Working solutions for peptide modification with ZEN-CMO

Solution	Components
ZEN-CMO stock solution	10 mg/ml in DMF
NHS stock solution	5 mg/ml in DMF
DCC stock solution	10 mg/ml in DMF
DMF	100% Dimethylformamide
CON stock solution	5 mg/ml in 0.1 M $NaHCO_3$ (pH 8.0)

Procedure for ZEN-CMO-CON (cr 1:50):

Substances	Molar cr
Protein : ZEN-CMO	1 : 50
Protein : NHS	1 : 75
Peptide : DCC	1 : 75

- Combine 125.7 µl ZEN-CMO with 111.0 µl NHS, 163.9 µl DMF and 99.5 µl DCC (= hapten solution). Keep this order!
- Wait 1 hour for activation (no stirring).
- Cool 1 ml CON stock solution in an ice bath with slow stirring. Add 500 µl of hapten solution and shake slowly.
- Incubate at 4°C over night on a shaker.
- Purify protein conjugates using PD-10 desalting columns.

2.2.3 Peptide modification

For this study a peptide with the sequence KRTLRR was modified with a cr 1:4. Based on the results of C. Stephan[91], it was expected that at this cr mainly peptides carrying one ZEN-CMO modification would be observed.

Working solutions:

Table 5 Working solutions for peptide modification with ZEN-CMO

Solution	Components
ZEN-CMO stock solution	10 mg/ml in DMF
NHS stock solution	5 mg/ml in DMF
DCC stock solution	10 mg/ml in DMF
DMF	100% Dimethylformamide
KRTLRR stock solution	1.86 mg/ml in 0.1 M $NaHCO_3$ (pH 8.0)

Procedure for KRTLRR modification (cr 1:4):

Table 6 Molar ratios for the different substances for coupling

Substances	Molar ratios
Peptide : ZEN-CMO	1 : 4
Peptide : NHS	1 : 4,4
Peptide : DCC	1 : 4,4

- Combine 175.5 µl ZEN-CMO with 113.6 µl NHS, 109.1 µl DMF and 101.8 µl DCC. Keep this order! (= hapten solution)
- Wait 1 hour for hapten activation (no stirring).
- Cool 500 µl KRTLRR stock solution (0.93 µg peptide) in an ice bath with slow stirring. Add 500 µl of hapten solution and shake slowly.
- Incubate at 4°C over night on a shaker.

2.3 In-solution protein digestion

2.3.1 Digestion with immobilized trypsin

Working Solutions:

Table 7 Working solutions for digestion with immobilized trypsin

Solution	Components
CON stock solution	1 mg/ml CON in UHQ water
Digestion buffer	40 mM NH_4HCO_3 (pH 8.5), 1.2 M urea, 0.4 M thiourea
Reducing solution	400 mM DTT (in 40 mM NH_4HCO_3)
Alkylation solution	800 mM IAA (in 40 mM NH_4HCO_3)
Washing solution	40 mM NH_4HCO_3
Protease	Immobilized TPCK (supplied as a 50% slurry containing glycerol and 0.05% sodium azide)

Procedure:

- Add 77 µl of the CON stock solution (equates to 77 µg of protein) to 500 µl digestion buffer in a 1.5 ml sample tube.
- For the reduction step add 50 µl reducing solution. Vortex and put the tubes on a thermomixer at 37°C and 850 rpm for 60 minutes.
- Add 50 µl freshly prepared alkylation solution, vortex and leave the tubes in the dark for at least 30 minutes at room temperature.
- Add again 50 µl reducing solution and vortex.
- Pipette 350 µl of the protein solution into a 10 kDa centrifugal filter and centrifuge at 14000 x g until the complete solution has passed the membrane (about 15 minutes). Discard the flow through. Repeat with the remaining solution.
- Wash the retentate with 100 µl washing solution by centrifuging at 14000 x g for 5 minutes. Discard the flow through and repeat this step.

- Dissolve the proteins on the centrifugal filter membrane in 2 x 100 µl digestion buffer and transfer the solution into a new sample tube. Add 300 µl digestion buffer.
- Wash 0.1 ml of the immobilized TPCK trypsin 3 times with 500 µl of the digestion buffer by vortexing. Separate the gel from the buffer after each wash by centrifugation (use spin-centrifuge). Discard the buffer after each washing step.
- Suspend the beads in 0.2 ml of the digestion buffer.
- Combine the protein solution with the protease solution.
- Digest over night (~18 hours) at 37°C on a thermomixer (850 rpm).
- After digestion the agarose beads have to be removed. Centrifuge the solution in 10 kDa spinfilters (14000 x g, 10 min). Recover the flow through. Discard the beads.

2.3.2 Digestion with trypsin/LysC mix

Working Solutions:

Table 8 Working solution for digestion with trypsin/lysC mix

Solution	Components
CON stock solution or ZEN-CMO-CON stock solution	1 mg/ml CON in UHQ water 1 mg/ml ZEN-CMO-CON in 0.01 M PBS buffer
Digestion buffer	40 mM NH_4HCO_3 (pH 8.5), 1.2 M urea, 0.4 M thiourea
Reducing solution	400 mM DTT (in 40 mM NH_4HCO_3)
Alkylation solution	800 mM IAA (in 40 mM NH_4HCO_3)
Washing solution	40 mM NH_4HCO_3
Trypsin/LysC mix	0.2 µg/µl resp. 40 ng/µl trypsin/LysC mix (in 50 mM acetic acid)

Procedure:

- Add 77 µl of the CON stock solution (equates to 77 µg of protein) to 500 µl digestion buffer in a 1.5 ml sample tube.

- For the reduction step add 50 µl reducing solution. Vortex and put the tubes on a thermomixer at 37°C and 850 rpm for 60 minutes.
- Next add 50 µl freshly prepared 800 mM alkylation solution, vortex and leave the tubes in the dark for at least 30 minutes at room temperature.
- Add again 50 µl reducing solution and vortex.
- Pipette 350 µl of the solution into a 10 kDa centrifugal filter and centrifuge at 14000 x g until the complete solution has passed the membrane (about 15 minutes). Discard the flow through. Repeat with the remaining solution.
- Wash the retentate with 100 µl washing solution by centrifuging at 14000 x g for 5 minutes. Discard the flow through and repeat this step.
- Dissolve the protein on the centrifugal filter membrane in 2 x 100 µl digestion buffer and put the solution into a new sample tube.
- Add trypsin/LysC mix (protein to protease ratio (w/w) = 25:1).
- Add enough digestion buffer to get a final reaction volume of 500 µl total.
- Digest over night at 37°C on a thermomixer (850 rpm).

2.4 Desalting

After protein digestion the samples had to be desalted to ensure their compatibility with (n)ESI-MS. Desalting by C_{18} spin columns (Thermo scientific, Rockford, IL, USA) was performed for all samples. For the samples measured by nLC-MS/MS a second desalting step using C_{18} ZipTips® (Milipore, Bedford, MA, USA) was conducted to completely assure the purity of the sample. Since high sample loss occurs during the desalting procedure, desalting over C_{18} ZipTips® was omitted after the first experiments, but was later performed again in the hope to reduce system abnormalities observed for the nLC instrumentation (see chapter 2.5.1). Desalting with C_{18} ZipTips® was never performed for UPLC-MS/MS measurements.

2.4.1 Desalting with C_{18} spin columns

Working Solutions:

Table 9 Working solutions for desalting with C_{18} spin columns

Solution	Components
Activation solution	50% ACN in UHQ water / 0.05% FA
Equilibration solution	5% ACN in UHQ water / 0.05% FA
Washing solution	UHQ water / 0.05% FA
Elution solution	50% ACN in UHQ water / 0.05% FA

Procedure:

Note: All centrifugation steps in this procedure are conducted at 1500 x g for 1 minute.

- Remove the caps from the C_{18} spin column and put it into a fresh sample tube.
- Pipette 200 µl activation solution onto the column and use it to wash down C_{18} material adhering to the walls of the column. Centrifuge. Discard flow through and repeat this step.
- Add 200 µl of equilibration solution and centrifuge. Discard the flow through. Repeat this step.
- Pipette 150 µl sample solution onto the column. Centrifuge. Recover the flow through and transfer it again onto the column, to ensure complete sample binding. Centrifuge and discard the flow through. Next wash the column by adding 150 µl of washing solution and centrifuge again. Repeat these steps until the whole sample solution has been bound to the column.
- Add 150 µl of washing solution to the column. Centrifuge and discard the flow through. Repeat this 5 times.
- Finally place the column into a new sample tube. Add 150 µl of elution solution. Centrifuge. Save the flow through. Put the column again into a new sample tube and elute again with 150 µl of elution solution. Combine the 2 eluates and dry the sample gently in a vacuum evaporator (heating turned on).

2.4.2 Desalting with C_{18} ZipTips®

This step is performed after desalting by C_{18} spin columns. The dried samples are redissolved in 10 µl 5% ACN in UHQ water / 0.05% FA.

Working Solutions:

Table 10 Working solutions for desalting with C_{18} ZipTips®

Solution	Components
Activation solution	100% ACN / 0.05% FA
Equilibration solution	5% ACN in UHQ water / 0.05% FA
Washing solution	UHQ water / 0.05% FA
Elution solution	50% ACN in UHQ water / 0.05% FA

Procedure:

- Wet the C_{18} material in the ZipTip® 3 times with 10 µl of activation solution (aspirate solution and discard). The C_{18} material in the tip must not run dry during the whole procedure.
- Equilibrate the material 3 times with the equilibration solution.
- Bind the peptides to the C_{18} material by repeatedly aspirating the sample solution.
- Wash the material 3 times with the washing solution.
- Finally elute the peptides with 10 µl of elution solution.
- Dry the samples gently in a vacuum evaporator (heating turned on).

2.4.3 Preparing the desalted sample for nLC-MS/MS measurement

Before measuring the samples on the nLC-QIT-mass-spectrometer, they were thoroughly dissolved in 10 µl 5% ACN in UHQ water / 0.05% FA by vortexing and putting them in an ultrasonic bath for approximately 10 seconds. The dissolved samples were then diluted 1:10 with 5% ACN in UHQ water / 0.05% FA. This would result in a sample concentration of 10 pmol/µl if

all protein was conveyed from digestion to MS measurement. However since C_{18} spin columns have a sample binding capacity of 30 µg and the C_{18} ZipTips® of only 5 µg, high sample losses are expected during desalting and the actual concentration is estimated to be way lower (max. 3.9 pmol/µl for samples only desalted using C_{18} spin columns and 649.4 fmol/µl for samples desalted additionally over C_{18} ZipTips®).

2.5 Chromatography and mass spectrometry

2.5.1 Nano HPLC separation

Instrument and column parameters are described in chapter 2.1.2.

Table 11 Chromatography parameters used in nLC

Parameter	Setting
Flow rate	0.250 µl/min
Injection volume	1 µl
Temperature	25°C
Mobile Phase A	UHQ water / 0.05% FA
Mobile Phase B	ACN / 0.05% FA

For peptide identification 2 different gradients were used.

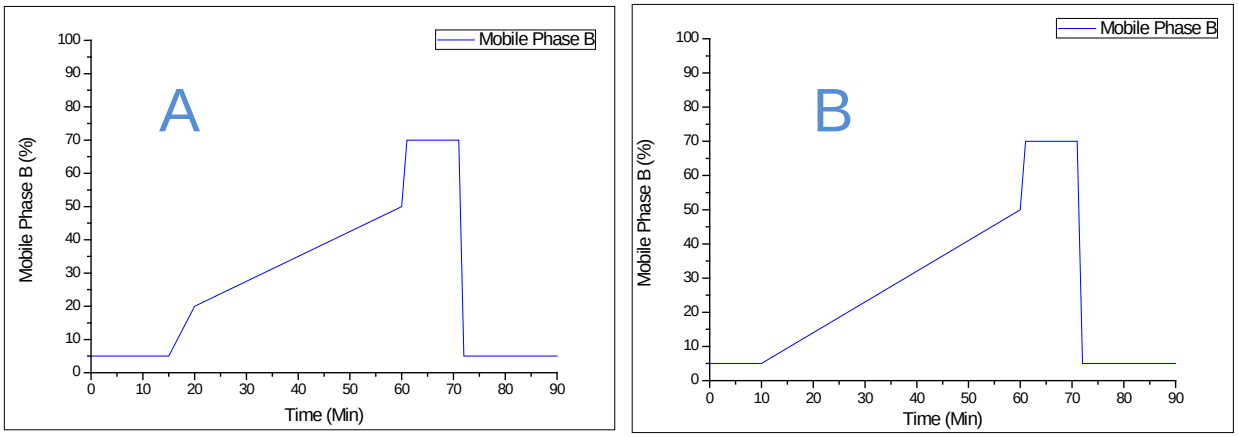


Figure 24 Gradients used for nano-HPLC measurements. The graphs show the percentage of mobile phase B over time. Gradient A is shown on the left. Gradient B is shown on the right.

CON digests were first measured using gradient A. For better comparison of the results using "TheorChromo online version 1.0" (see chapter 3.4), gradient B was later used for peptide identification of ZEN-CMO-CON digests (see Figure 24).

To ensure binding of the peptides to the stationary phase, both gradients have a 10 minute "hold" state at 5% mobile phase B before elution starts. Since the peptides were purified on a C_{18} spin column and eluted with 50% ACN in UHQ water / 0.05% FA, the gradient increases to max. 50% of mobile phase B. After this a 10 min plateau of 70% mobile phase B follows to ensure that all substances are eluted from the chromatographic column. Finally the gradient decreases to 5% mobile phase B to reequilibrate the system before the next measurement.

Troubleshooting

Interestingly when measuring peptides derived from ZEN-CMO-CON digests, abnormalities in the instruments performance could be observed (see Figure 25). The pressure started to increase soon after injection by about 30-40 bar. After multiple separations the pressure instability worsened and did also occur during the entire chromatographic run (90 min).

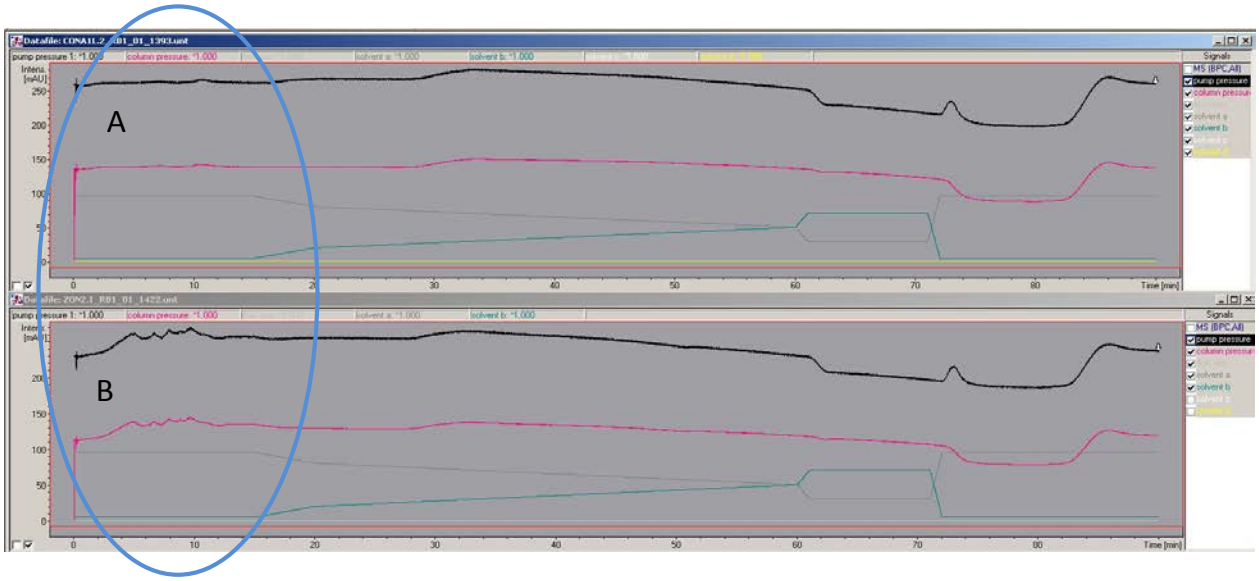


Figure 25 Comparison of the pressure observed during chromatographic separation of an unmodified CON (A) and a modified ZEN-CMO-CON (B) sample. The pump pressure is displayed in black. The column pressure is displayed in pink.

ZEN-CMO-CON digests for identification of modification sites were therefore measured under varying set-ups. To solve the pressure instability, different approaches were tested:

1. Increasing the amount of FA in the solvents to 1% to improve solubility

2. Further dilution of the samples in case the samples were to high concentrated (sample concentration ranged from 3.9 pmol/µl to 324.7 fmol/µl)
3. Increasing the initial concentration of ACN to 15% and 20%
4. Switching mobile phase B to 65% ACN / 35% 2-propanol / 0.05% FA for better column regeneration

None of these approaches showed significant improvement. Only after changing the column to a new one the pressure instability disappeared. It is assumed that LC-MS measurements of ZEN-CMO-CON digests reduce the shelf life of the chosen nano-column significantly. This has to be taken into account for future experiments.

2.5.2 nESI-QIT settings

For nESI-QIT measurements the following settings were applied (Table 12):

Table 12 Settings applied for nESI-QIT measurements

Functional unity	Parameter	Setting
Source	Nebulizer	2.0 psi
	Dry gas	0.5 l/min
	Dry temp	200°C
	Polarity	positive
Trap	Scan mode	Standard enhanced
	Smart target	200000
	Max accu time	200 ms
	Scan area	300 – 1500 m/z
	Ramp range	from – 4500 V to – 1500 V
	MS/MS frag ampl	1 V
	Collision gas	Helium

Peptides are measured in positive ion mode. The standard enhanced scan mode was applied, which is a high-resolution scan mode (0.4 FWHM / m/z) with average scan speed (8100 m/z / s). “Smart target” describes the maximum number of ions accumulated in the QIT during one cycle. Whichever limit is reached first, decides when an accumulation cycle is finished. MS/MS was performed with a ramping range from -4500 V to -1500 V and a fragmentation amplitude of 1 V.

2.5.3 Nexera UPLC separation

The overall settings for UPLC separations can be taken from Table 13.

Table 13 Chromatography parameters applied on Nexera UPLC

Parameter	Setting
Flow rate	0.5 ml/min
Mobile Phase A	UHQ water / 0.05% FA
Mobile Phase B	ACN / 0.05% FA
Oven temperature	40°C
Autosampler cooler temp	15°C
Sampling speed	5.0 µl/s

Before UPLC-MS/MS measurements the ZEN-CMO-CON digests were usually dissolved in 50 µl 5% ACN in UHQ water / 0.05% FA, resulting in a concentration of 20 pmol/µl, assuming that all protein was conveyed from digestion to MS measurement. Again this is unlikely due to the binding capacity of the desalting equipment. For UPLC-MS/MS measurements samples were desalted using C_{18} spin columns exclusively. Since the maximum binding capacity of these columns is limited to peptides derived from 30 µg of protein, the actual concentration is 7.8 pmol/µl. 10 µl of sample solution were usually injected for measurements, resulting in an estimated absolute amount of 78 pmol.

The ZEN-CMO modified peptide KRTLRR was diluted to a concentration of 0.01 µg/µl (12.1 pmol/µl). 5 µl were injected, which is a total amount of 60.5 pmol. Since the peptide was not purified after modification, free ZEN-CMO was still included in the solution. This was used to perform product ion scan on the free ZEN-CMO to determine product ions for further experiments. The amount of free ZEN-CMO is unknown.

Gradient C (Figure 26) was used for measurements of ZEN-CMO-CON digests. Since the modified peptides are more hydrophobic than the unmodified peptides (see chapter 3.4) they have a stronger interaction with the column and elute at a higher percentage of organic solvent. Thus the hold of mobile phase B at 5% to bind peptides was omitted and the gradient increased immediately from 5 to 50% within 45 minutes. After this the column is washed with 90% mobile phase B for 7 minutes and then the system is equilibrated back to 5% ACN in UHQ water /

0.05% FA. For method compatibility the same gradient was used for measurements of the unmodified peptides in a CON digest.

For measurements of the peptide KRTLRR and free ZEN-CMO a shorter gradient (D) of 20 minutes was used. Details for both gradients can be seen in Figure 26.

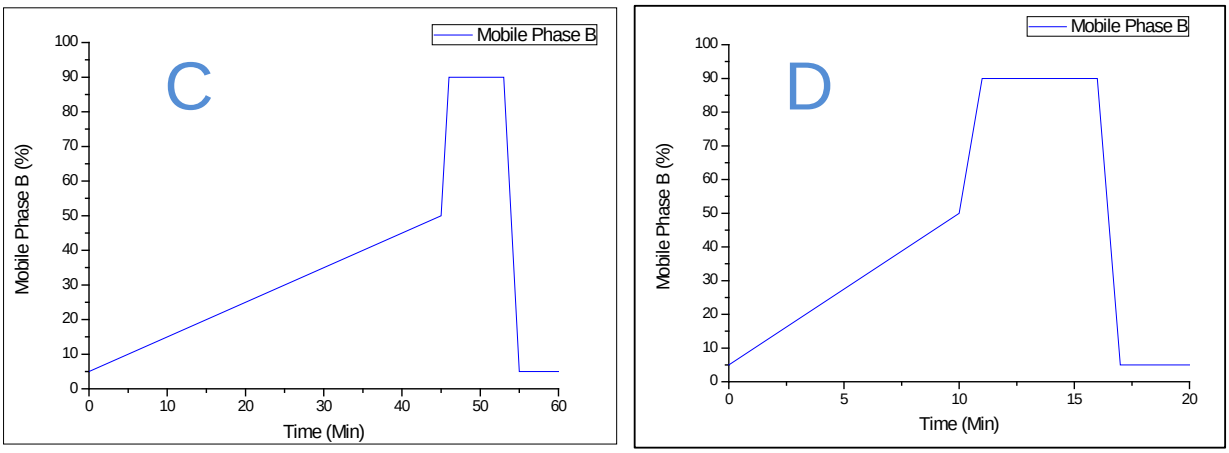


Figure 26 Mobile phase B gradients used for LC-ESI-QQQ measurements of ZEN-CMO-CON and CON digests (C) and the ZEN-CMO modified peptide KRTLRR (D) on the Nexera system.

2.5.4 ESI-QQQ settings

Table 14 shows the general settings applied for ESI-QQQ measurements.

Table 14 General settings on the LC-MS 8030plus

Parameter	Setting
Nebulizing gas flow	3 l/min
DL temperature	250°C
Heat block temperature	400°C
Drying gas flow	15 l/min
Collision gas	Argon

Precursor and product ions differed depending on the scan method. Various fragmentation energies (-15 V to -35 V) were applied for fragmentation optimization.

Settings product ion scan:

The precursor for product ion scan was chosen according to the measured compound. For peptide KRTLRR product ion scan was performed using the precursors m/z 602 ($[M+2H]^{2+}$) and m/z 402 ($M+3H]^{3+}$). Product ion scan of free ZEN-CMO was performed using m/z 392 as precursor ($[M+H]^{+}$). The applied scan range was 100 – 1500 m/z.

Settings neutral loss scan:

Neutral loss scan was performed by checking for the loss of the ZEN-CMO molecule, which results in a loss of 315.15 Da. Therefore constant mass differences of m/z 157.6 ($[M+2H]^{2+}$) and m/z 105.0 ($[M+3H]^{3+}$) were measured. The scan range was set from m/z 115 resp. m/z 167.6 to m/z 1500.

Settings precursor ion scan:

Precursor ion scan was performed using m/z 203 as product ion, which is a fragment of the ZEN molecule. Scan range was set from m/z 100 to m/z 1500.

Settings MRM quantification method:

The actual method for quantification is a MRM method. All modified ions identified on the nLC instrument were chosen as precursor ions. The MRM transitions were all set to the same product ion, m/z 203. A CE of -30 V was applied and the dwell time was set to 100 ms. Time frames were set for each peptide to keep dwell times low and to increase the number of measurement points per MRM transition. A detailed summary of the method is shown in Table 15.

Table 15 MRM transitions plus time frames in the quantification method for ZEN-CMO modifications

Event	Precursor ion	Product ion	Time frame
1	463.6	203.0	18.7 – 20.1
2	467.2	203.0	20.0 – 21.2
3	504.7	203.0	20.3 – 21.4
4	519.7	203.0	20.3 – 21.4
5	603.3	203.0	20.4 – 21.5
6	589.3	203.0	21.6 – 22.8
7	487.8	203.0	22.9 – 24.2
8	707.9	203.0	23.4 – 24.5

9	713.3	203.0	23.5 – 24.8
10	536.8	203.0	24.8 – 26.5
11	852.9	203.0	25.5 – 26.6
12	869.4	203.0	25.6 – 26.7
13	603.3	203.0	25.7 – 26.9
14	620.8	203.0	25.8 – 27.1
15	945.5	203.0	26.1 – 27.3
16	630.6	203.0	26.2 – 27.5
17	905.4	203.0	26.4 – 27.4
18	1013.0	203.0	26.5 – 27.6
19	853.9	203.0	27.2 – 28.4
20	704.8	203.0	27.4 – 28.4
21	881.4	203.0	27.6 – 28.6
22	653.8	203.0	28.5 – 29.7
23	598.3	203.0	29.2 – 30.5
24	568.3	203.0	29.7 – 31.0
25	752.4	203.0	29.7 – 32.0
26	699.3	203.0	31.2 – 32.2
27	875.4	203.0	31.7 – 32.8
28	904.4	203.0	33.4 – 34.5
29	889.9	203.0	34.0 – 35.1
30	954.5	203.0	35.1 – 36.1
31	1076.0	203.0	35.8 – 37.0
32	933.8	203.0	39.9 – 41.2
33	1060.5	203.0	41.1 – 44.0

Settings for identification of unmodified peptides:

For the determination of the degree of modification, the unmodified peptides were searched. The doubly as well as the triply charged peptide were measured, each with 2 corresponding fragment ions. For fragmentation, ions of significant intensity in nLC-QIT data were chosen considering comparable ionization efficiencies. Time windows were chosen according to single ion monitoring (SIM) data and peptide RT difference between nLC and UPLC measurements. 78 pmol were measured (Table 16).

Table 16 Settings for MRM of unmodified peptides, 78 pmol

Sequence	m/z Precursor ions	m/z Product ions	Time frame (min)
K.DGKGDVAFVK.H	518.3^{2+}, 345.9^{3+}	$y_7 = 735.5$, $y_9 = 460.7$	0 – 15
R.KDQLTPSPR.E	521.3^{2+}, 347.9^{3+}	$y_4 = 456.2$, $y_8 = 913.5$	0 – 15
R.KDSNVNWNNLK.G	666.3^{2+}, 444.6^{3+}	$b_6 = 657.8$, $y_6 = 788.5$	0 – 15
K.SDFHLFGPPGKK.D	665.4^{2+}, 443.9^{3+}	$y_4 = 429.2$, $y_{10} = 564.3$	0 – 15
R.IQWCAVGKDEK.S	667.3^{2+}, 445.2^{3+}	$b_6 = 1058.6$, $y_9 = 576.3$	7 – 12
K.KGTEFTVNDLQGK.T	718.9^{2+}, 479.6^{3+}	$y_6 = 674.3$, $b_7 = 763.4$	8 – 14
K.FMMFESQNKDLLFK.D	889.4^{2+}, 593.3^{3+}	$y_9 = 1092.6$, $y_4 = 520.4$	19 - 29
R.SAGWNIPIGTLLHR.G	767.9^{2+}, 512.3^{3+}	$y_8 = 906.6$, $b_{12} = 612.3$	22 – 24
K.EFLGDKFYTVISSLK.T	874.0^{2+}, 583.0^{3+}	$y_9 = 1057.7$, $y_8 = 910.5$	24 – 29

In the second measurement of unmodified peptides, MRM transitions per time window were reduced and sample amount was increased to 300 pmol (Table 17).

Table 17 Settings for MRM of unmodified peptides for measurement of 300 pmol

Sequence	m/z Precursor ions	m/z Product ions	Time frame (min)
K.DGKGDVAFVK.H	518.3^{2+}, 345.9^{3+}	$y_7 = 735.5$, $y_9 = 460.7$	0 – 15
R.KDQLTPSPR.E	521.3^{2+}, 347.9^{3+}	$y_4 = 456.2$, $y_8 = 913.5$	0 – 5
K.SDFHLFGPPGKK.D	665.4^{2+}, 443.9^{3+}	$y_4 = 429.2$, $y_{10} = 564.3$	5 – 10
R.IQWCAVGKDEK.S	667.3^{2+}, 445.2^{3+}	$b_6 = 1058.6$, $y_9 = 576.3$	9 – 11
K.KGTEFTVNDLQGK.T	718.9^{2+}, 479.6^{3+}	$y_6 = 674.3$, $b_7 = 763.4$	10 – 12
K.FMMFESQNKDLLFK.D	889.4^{2+}, 593.3^{3+}	$y_9 = 1092.6$, $y_4 = 520.4$	21 - 30
R.SAGWNIPIGTLLHR.G	767.9^{2+}, 512.3^{3+}	$y_8 = 906.6$, $b_{12} = 612.3$	22 – 24
K.EFLGDKFYTVISSLK.T	874.0^{2+}, 583.0^{3+}	$y_9 = 1057.7$, $y_8 = 910.5$	24 – 28

2.6 Data interpretation

2.6.1 Protein and peptide identification

nLC-QIT data was investigated using “DataAnalysis 3.2” (Bruker) and “BioTools 3.2” (Bruker).

Compounds with a S/N of at least 5 were exported in Data Analysis and opened in BioTools. A maximum of 250 compounds was exported. Protein and peptide identification was performed by MASCOT search (Matrix Science, London, UK)[83] using the parameters shown in Table 18. Since the digestion with trypsin/LysC mix cleaves at the same positions as if sole trypsin was used, trypsin was selected as enzyme for all searches.

Table 18 Database search parameters for MASCOT search

Category	Search parameter
Taxonomy	Eukaryota
Database	NCBInr
Enzyme	Trypsin
Partials	1
Global modifications	Carbamidomethyl (C)
Variable modifications	Acetyl (N-term), Oxidation (M)
Mass tolerance	0.5 Da
MS/MS tolerance	0.5 Da
Charge state	1+, 2+ and 3+
Instrument	ESI-TRAP

Modified peptides were identified by using the “SequenceEditor” function in BioTools. The sequence of CON (see Figure 27) was edited so that all cysteines are carbamidomethylated. Oxidation was set as variable modification for methionine (Met). The ZEN-CMO modification was also introduced as possible modification for Lys, Arg, Thr and Ser, which leads to the loss of H_2O and a gain of $C_{20}H_{25}NO_7$. This results in a mass increase of 373.15 Da. An *in-silico* digest of the modified sequence was performed and matched to QIT measurement results. To confirm a match, MS/MS spectra were manually searched for ZEN-CMO reporter ions at m/z 283, 300, 301, 316 and 318.

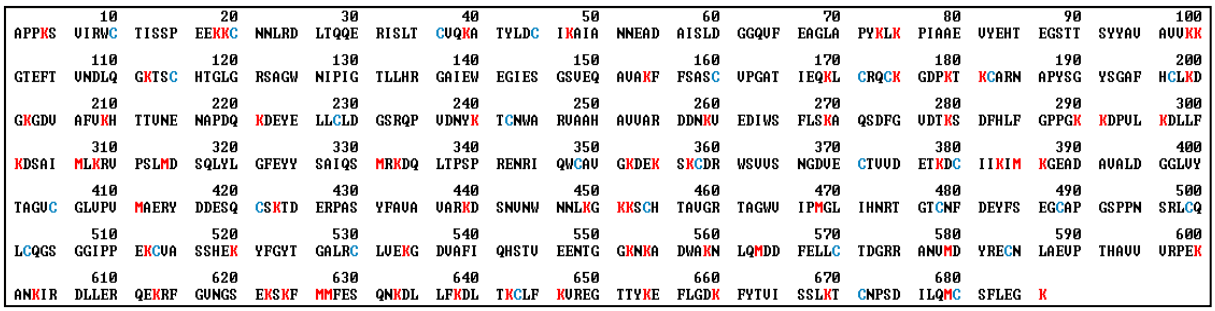


Figure 27 Sequence of CON used for identification of modification sites

Additional information on protein digestion results and fragmentation pattern was obtained via "Protein Prospector 5.14.1". (http://prospector.ucsf.edu, University of California, San Francisco).

2.6.2 Retention time simulation

For simulation of peptide RTs the freeware "TheorChromo 1.0" (www.theorchromo.ru)[92] was used. The chromatography parameters used are displayed in Table 19 and Table 20. Cysteines are selected as carboxyamidomethylated. For further information see chapter 3.4.

Table 19 Chromatography parameters for the simulation of RTs for nLC in "TheorChromo 1.0"

System parameter	Setting
Column length	150 mm
Column internal diameter	0.075 mm
Packing material pore size	100 A
Initial concentration of component B	5.0 %
Final concentration of component B	50.0 %
Gradient time	45.0 min
Delay time	28.0 min
Flow rate	0.00025 ml/min
ACN concentration in component A	0.0 %
ACN concentration in component B	100.0 %
Solid / mobile phase combination	RP/ACN + FA

Table 20 Chromatography parameters for the simulation of RTs for UPLC in "TheorChromo 1.0"

System parameter	Setting
Column length	100 mm
Column internal diameter	2.1 mm
Packing material pore size	80 A
Initial concentration of component B	5.0 %
Final concentration of component B	50.0 %
Gradient time	50.0 min
Delay time	2.0 min
Flow rate	0.5 ml/min
ACN concentration in component A	0.0 %
ACN concentration in component B	100.0 %
Solid / mobile phase combination	RP/ACN + FA

2.6.3 Interpretation of triple quadrupole measurements

UPLC-QQQ data was investigated manually in LabSolutions 5.6 (Shimadzu Kratos Analytical). Peak integration was performed and peak areas were summed up for final results.

3 Results and discussion

3.1 Sequence identification

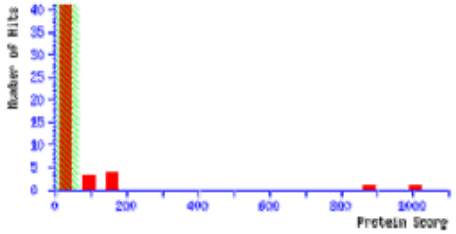
For this study the carrier protein CON had to be thoroughly characterized. At first the correct protein sequence was determined. ZEN-CMO-CON was digested as described in chapter 2.3.2 and the protein sequence was determined by MASCOT search.

MATRIX SCIENCE **Mascot Search Results**

```
User           : ES
Email          :
Search title   : A1
MS data file   : DATA.TXT
Database       : NCBInr 20150329 (63477871 sequences; 22673374932 residues)
Taxonomy       : Eukaryota (eucaryotes) (16689678 sequences)
Timestamp      : 3 Apr 2015 at 14:08:58 GMT
Protein hits   : gi|83754919  Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution
                 gi|1351295   RecName: Full=Ovotransferrin; AltName: Full=Allergen Gal d III; AltName: Full=Conalbumin; AltName: Full=
                 gi|678144816 Ovotransferrin, partial [Tauraco erythrolophus]
                 gi|704284139 PREDICTED: ovotransferrin-like [Eurypyga helias]
                 gi|677499351 Ovotransferrin, partial [Phoenicopterus ruber ruber]
                 gi|483501900 Ovotransferrin, partial [Anas platyrhynchos]
                 gi|136429    RecName: Full=Trypsin; Flags: Precursor [Sus scrofa]
                 gi|554561470 PREDICTED: cationic trypsin-3-like [Myotis brandtii]
                 gi|478512953 PREDICTED: anionic trypsin-like [Ceratotherium simum simum]
                 gi|205055    keratin K5 [Rattus norvegicus]
                 gi|594692390 PREDICTED: vesicle-trafficking protein SEC22c isoform X1 [Balaenoptera acutorostrata scammoni]
                 gi|6981420   anionic trypsin-1 precursor [Rattus norvegicus]
                 gi|585651800 PREDICTED: keratin, type II cytoskeletal 1b [Elephantulus edwardii]
                 gi|167375825 hypothetical protein [Entamoeba dispar SAW760]
                 gi|260941614 hypothetical protein CLUG_04988 [Clavispora lusitaniae ATCC 42720]
                 gi|255082284 Multidrug/Oligosaccharidyl-lipid/Polysaccharide flippase [Micromonas sp. RCC299]
                 gi|385303998 putative srpk1-like protein kinase [Brettanomyces bruxellensis AWRI1499]
                 gi|682393290 hypothetical protein V499_07278 [Pseudogymnoascus pannorum VKM F-103]
                 gi|1708509   RecName: Full=Ovoinhibitor; Flags: Precursor [Gallus gallus]
                 gi|635361019 unnamed protein product [Albugo candida]
```

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 64 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help

Significance threshold p< 0.05 — Max. number of hits 20

Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Sort unassigned Decreasing Score — Require bold red

Preferred taxonomy: All entries

1. gi|83754919 Mass: 77518 Score: 1008 Matches: 31(4) Sequences: 18(4) emPAI: 0.25
Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
70	524.2000	1046.3854	1046.5185	-0.1331	0	46	3.6	1		K.YFGYTGALR.C
71	529.2500	1056.4854	1056.5855	-0.1001	0	37	25	1		K.FYTVISSLK.T
93	601.2300	1200.4454	1200.5928	-0.1473	0	(23)	5.9e+02	4		K.SDFHLFGPPGK.K
27	401.1700	1200.4882	1200.5928	-0.1046	0	38	19	1		K.SDFHLFGPPGK.K
95	612.3000	1222.5854	1222.6234	-0.0379	0	56	0.28	1		K.VEDIWSFLSK.A
110	654.7800	1307.5454	1307.6358	-0.0903	0	74	0.0041	1		K.GTEFTVNDLQGK.T
67	512.2300	1533.6682	1533.8416	-0.1734	0	46	3	1		R.SAGWNIPIGTLIER.G
126	767.8700	1533.7254	1533.8416	-0.1161	0	(42)	7.1	1		R.SAGWNIPIGTLIER.G
137	821.3600	1640.7054	1640.7868	-0.0814	0	49	1.6	1		K.FFSASCVPGATIEQK.L
138	826.8200	1651.6254	1651.8318	-0.2064	0	(31)	1e+02	1		K.TDERPASYFAVAVAR.K
78	551.5900	1651.7482	1651.8318	-0.0836	0	(43)	6.3	2		K.TDERPASYFAVAVAR.K
79	551.6000	1651.7782	1651.8318	-0.0536	0	59	0.17	1		K.TDERPASYFAVAVAR.K
140	836.3000	1670.5854	1670.7511	-0.1657	0	52	0.95	1		R.NAPYSGYSGAFECLK.D
146	848.3700	1694.7254	1694.8152	-0.0897	1	55	0.53	1		R.DDNKVEDIWSFLSK.A
83	565.9500	1694.8282	1694.8152	0.0130	1	(38)	23	1		R.DDNKVEDIWSFLSK.A
89	582.9100	1745.7082	1745.9240	-0.2158	1	22	7.9e+02	4		K.EFLGDKFYTVISSLK.T
163	889.3700	1776.7254	1776.8579	-0.1324	1	54	0.75	1		K.FHHFESQNKDLLFK.D
171	913.8000	1825.5854	1825.7975	-0.2120	0	(50)	1.5	1		K.NLQMDDFELLCTDGR.R
172	913.8700	1825.7254	1825.7975	-0.0720	0	(54)	0.64	1		K.NLQMDDFELLCTDGR.R
174	921.8500	1841.6854	1841.7924	-0.1070	0	58	0.26	1		K.NLQMDDFELLCTDGR.R + Oxidation (M)
204	980.3900	1958.7654	1958.9585	-0.1931	0	(31)	1.2e+02	1		R.GAIEWEGIESGSVEQAVAK.F
205	980.4500	1958.8854	1958.9585	-0.0731	0	78	0.0025	1		R.GAIEWEGIESGSVEQAVAK.F
109	654.0400	1959.0982	1958.9585	0.1397	0	(61)	0.098	1		R.GAIEWEGIESGSVEQAVAK.F
111	661.7800	1982.3182	1981.8986	0.4196	1	42	6.5	1		K.NLQMDDFELLCTDGRR.A
214	1000.3700	1998.7254	1998.8849	-0.1595	0	77	0.0033	1		K.TCNPSDILQMCSFLEGK.-
215	1000.3800	1998.7454	1998.8849	-0.1395	0	(45)	4.8	1		K.TCNPSDILQMCSFLEGK.-
221	1008.3200	2014.6254	2014.8798	-0.2544	0	(50)	1.5	1		K.TCNPSDILQMCSFLEGK.- + Oxidation (M)
222	1008.3700	2014.7254	2014.8798	-0.1544	0	(50)	1.3	1		K.TCNPSDILQMCSFLEGK.- + Oxidation (M)
249	1275.4200	2548.8254	2549.0224	-0.1969	0	44	4.6	1		R.TGTCNFDEYFSEGCAPGSPPNSR.L
250	1317.5700	2633.1254	2633.2973	-0.1718	0	122	7.9e-08	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
156	878.8000	2633.3782	2633.2973	0.0809	0	(50)	1.6	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L

Figure 28 Example of a MASCOT search result of a CON digest

Information on CON is given in chapter 1.2.2. As described natural variations of the sequence are possible. The signal peptide was never identified in any MS/MS experiments, which was expected, since it is not part of the mature protein. Figure 28 shows an example for a MASCOT search result of sample A1.

Regularly the highest scored hit that could be achieved in MASCOT searches was “Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution” (NCBInr entry: gi|83754919).

This sequence is almost identical to the sequence of ovotransferrin (UniprotKB entry: P02789). The differences are the absence of the signal peptide, the natural variation for position 83, which is an exchange from Ala to Val, and a possible exchange on position 152 from Leu to Ile.

A unique peptide for the protein with the sequence K.AIANNEADAISLDGGQ**V**FEAGLAPYK.L (AA [67–92]) was regularly identified, the doubly charged peptide ($[M+2H]^{2+}$ at m/z 1317.7) as well as the triply charged ion species ($[M+3H]^{3+}$ at m/z 878.8). The sequence containing Ala instead of Val could however never be identified.

The peptide R.SAGWNIPIGTL**I**HR.G (141 – 154) was also frequently identified. In this peptide sequence Leu is exchanged to Ile. This AA exchange could however not be verified, since Leu and Ile are isobaric AAs (133.1 Da) and show no difference in low energy CID experiments. This is however of less importance for this study. The peptide is not unique to this protein.

These informations on the protein sequence were taken into account for all further experiments.

3.2 Comparison of immobilized trypsin and trypsin/LysC mix

Protein digestion is a very important factor in a bottom-up approach. Therefore the goal was to optimize the digestion to gain the highest possible SC. SC is the percentage of a database protein´s sequence matched by peptides identified in the measurement. For this purpose two different proteases were tested for digestion: immobilized trypsin (Thermo Scientific) and a trypsin/LysC mix (Promega).

CON (Fluka) was in-solution digested 3 times with immobilized trypsin and 3 times with trypsin/LysC mix. Each digest was then measured in triplicate. Peptides were identified by MASCOT search and the SC per injection was calculated. The results were compared. Triplicate of measurement (technical replicate) represents variations in the measurement setup. Triplicates of digestion (methodic replicate) evaluate the digestion method and the sample preparation, both increasing overall variability.

Table 21 shows all ions that could be detected and identified by MASCOT search. MASCOT search results are attached in the appendix.

Table 21 Table of identified peptide sequences in CON digests using immobilized trypsin and trypsin/LysC mix. Methodic replicates are replicates of the full methods, from sample preparation to LC-MS measurement, while technical replicates refer to LC-MS replicates of one digest. a ,b and c are different measurements of the same digest. Ch. = charge, CAM = carbamidomethylation, OX = oxidation

		Immobilized trypsin									Trypsin / LysC mix								
		Methodic replicates									Methodic replicates								
		t.rep.1			t.rep.2			t.rep.3			t.rep.1			t.rep.2			t.rep.3		
m/z charge state	Sequence	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
401.2^{3+}	**K.SDFHLFGPPGK.K**	x	x	x	x		x	x	x	x	x	x	x	x		x	x	x	x
404.2^{2+}	**K.TCNWAR.V** 2: CAM(C)			x							x				x				

443.9^{3+}	**K.SDFHLFGPPGKK.D**												x	x		x		x
444.6^{3+}	**R.KDSNVNWNNLK.G**	x																
447.3^{2+}	**R.VAAHAVVAR.D**						x					x				x	x	x
474.8^{2+}	**R.ISLTCVQK.A** 5: CAM (C)											x		x		x	x	x
479.6^{3+}	**K.KGTEFTVNDLQGK.T**	x	x			x		x		x	x	x	x		x	x	x	x	x
492.2^{2+}	**K.ATYLDCIK.A** 6: CAM (C)	x	x	x	x	x		x	x	x	x		x	x		x	x	x	x
494.7^{2+}	**K.TSCHTGLGR.S** 3: CAM (C)						x												
512.3^{3+}	**R.SAGWNIPIGTLIHR.G**	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
512.8^{2+}	**R.RANVMDYR.E**											x					x	
518.3^{2+}	**K.DGKGDVAFVK.H**					x				x	x	x				x	x	x
521.3^{2+}	**R.KDQLTPSPR.E**				x	x				x						x	x	x
522.3^{3+}	**R.TAGWVIPMGLIHNR.T**									x		x	x	x	x	x	x	x
524.3^{2+}	**K.YFGYTGALR.C**	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
529.3^{2+}	**K.FYTVISSLK.T**	x	x	x	x	x	x	x	x	x	x						x	
534.3^{2+}	**K.AQSDFGVDTK.S**	x	x	x	x	x	x	x	x	x	x				x	x	x	x	
546.8^{2+}	**K.DLLFKDLTK.C**									x								
551.6^{3+}	**K.TDERPASYFAVAVAR.K**	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
557.9^{3+}	**R.NAPYSGYSGAFHCLK.D** 13: CAM (C)		x							x	x	x	x	x	x	x	x	x
565.9^{3+}	**R.DDNKVEDIWSFLSK.A**									x			x	x	x	x	x	x
581.3^{2+}	**K.FMMFESQNK.D**				x	x		x	x	x	x	x				x	x	
583.0^{3+}	**K.EFLGDKFYTVISSLK.T**									x	x	x		x	x	x	x	x
589.3^{2+}	**K.FMMFESQNK.D** 2: OX (M)							x										
598.6^{3+}	**K.FMMFESQNKDLLFK.D** 3: OX (M)									x		x						
601.3^{2+}	**K.SDFHLFGPPGK.K**		x		x			x		x	x	x	x				x	x
602.3^{2+}	**K.DSNVNWNNLK.G**								x							x	x	
618.8^{2+}	**R.WCTISSPEEK.K** 2: CAM (C)	x	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x

630.3^{3+}	**K.GDVAFIQHSTVEENTGGK.N**										x								
654.8^{2+}	**K.GTEFTVNDLQGK.T**	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
661.6^{3+}	**K.NLQMDDFELLCTDGRR.A** 11: CAM (C)										x		x		x	x		x	
666.3^{2+}	**R.KDSNVNWNNLK.G**										x	x	x				x		
667.3^{2+}	**R.IQWCAVGKDEK.S** 4: CAM (C)					x					x	x	x				x	x	x
683.4^{3+}	**R.ECNLAEVPTHAVVVRPEK.A** 2:CAM (C)	x	x	x	x	x	x		x		x	x	x	x	x	x	x	x	x
718.9^{2+}	**K.KGTEFTVNDLQGK.T**						x				x	x							
767.9^{2+}	**R.SAGWNIPIGTLIHR.G**	x	x	x	x	x	x	x			x	x	x	x	x	x	x	x	x
782.9^{2+}	**R.TAGWVIPMGLIHNR.T**										x		x		x		x	x	x
821.4^{2+}	**K.FFSASCVPGATIEQK.L** 6: CAM (C)	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x
822.4^{2+}	**R.LCQLCQGSGGIPPEK.C** 2: CAM (C), 5: CAM (C)	x	x	x		x			x	x	x	x	x		x	x	x		x
826.9^{2+}	**K.TDERPASYFAVAVAR.K**										x	x	x				x		x
836.4^{2+}	**R.NAPYSGYSGAFHCLK.D** 13: CAM (C)			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
848.4^{2+}	**R.DDNKVEDIWSFLSK.A**										x	x	x	x	x	x	x	x	x
850.7^{3+}	**R.TGTCNFDEYFSEGCAPGSP PNSR.L** 4: CAM (C), 14: CAM (C)										x	x	x					x	x
874.0^{2+}	**K.EFLGDKFYTVISSLK.T**										x	x	x	x	x	x	x	x	x
878.8^{3+}	**K.AIANNEADAISLDGGQVFEA GLAPYK.L**		x	x	x		x			x		x			x			x	x
889.4^{2+}	**K.FMMFESQNKDLLFK.D**										x	x	x		x	x	x	x	x
913.9^{2+}	**K.NLQMDDFELLCTDGR.R** 11: CAM (C)	x	x	x	x	x	x	x	x	x		x	x		x	x	x	x	x
935.4^{3+}	**K.HTTVNENAPDQKDEYELLCL DGSR.Q** 19: CAM (C)										x		x		x		x	x	x
980.5^{3+}	**R.GAIEWEGIESGSVEQAVAK. F**	x	x	x	x	x	x	x	x	x	x	x	x				x	x	
992.0^{2+}	**K.NLQMDDFELLCTDGRR.A** 11: CAM (C)												x						

1000.4^{2+}	**K.TCNPSDILQMCSFLEGK.-** 2: CAM (C), 11: CAM (C)	x	x	x	x	x	x	x		x	x	x	x		x	x	x	x	x
1054.9^{3+}	**K.IMKGEADAVALDGGLVYTAGVCGLVPVMAER.Y** 22: CAM (C)											x			x			x	
1275.5^{2+}	**R.TGTCNFDEYFSEGCAPGSPPNSR.L** 4: CAM (C), 14: CAM (C)									x							x	x	x
1317.7^{2+}	**K.AIANNEADAISLDGGQVFEAGLAPYK.L**	x	x						x							x		x	

As shown in Table 21, a total of 54 ions could be detected representing 40 peptides out of 155 possible tryptic peptides (min 3 AA and maximum 1 missed cleavage). With respect to the technical repeatability it was found that some ions were detected rather frequently, while others could rarely be found – if not just once. This is influenced by the ionization efficiency of the different peptides, especially in the case of co-eluting peptides. In total 33 ions were detected in immobilized trypsin digests and 51 in methodic replicates of the trypsin/LysC mix. Therefore it seems that the digestion with trypsin/LysC mix was more efficient. Only 2 ions were detected in every measurement of both approaches (R.SAGWNIPIGTLIHR.G and K.YFGYTGALR.C). 17 ions were detected in at least 5 out of the 9 injections per methodic replicate, which means that these peptides may have easily accessible cleavage sites. In the technical replicates of trypsin/LysC mix 10 ions could be detected in every measurement, while for the immobilized trypsin 8 ions were detected every time in every digest. 21 ions, corresponding to 15 different peptides, could only be detected in the digests using trypsin/LysC mix. 3 ions were only found in the digests of immobilized trypsin. However latter were only found once. This observation was rather typical for the immobilized trypsin, since in total 10 ions were identified only once in all experiments (30.3% of detected ions). For the trypsin/LysC mix this was observed only for 3 ions (5.9%). Most peptides can be found doubly or triply charged. Higher charged peptides were not included in the search, since resolution of the QIT is too low to determine the accurate charge state for molecular weight assignment, and hence peptide identification. No singly charged ions were detected, which is common for ESI-MS measurements of tryptic peptides. The average SC for both methodic replicates can be seen in Table 22.

The average SC for CON per injection for the digests with immobilized trypsin and trypsin/LysC mix was calculated and compared. Since the signal peptide could not be found in any digestion experiments and also previous studies concluded that it was no longer attached to the protein

(see diploma thesis of C. Stephan[91]), the 19 AAs forming the signal peptide were excluded from calculating the SC. The mean value of the results for digests with immobilized trypsin was 33.2% SC, while the trypsin/LysC mix digests gave a mean value of 48.0% SC. Thus a higher SC could be achieved by using the trypsin/LysC mix for protein digestion. It was however interesting to observe that the standard deviation was 2.7% for immobilized trypsin results and 10.7% for trypsin/LysC results. These results could lead to the conclusion that digestion with immobilized trypsin has a higher reproducibility than digestion with trypsin/LysC. It should however be noticed that the measurements of the same technical replicate also gave highly different results. Especially the technical replicate no. 2 for trypsin/LysC mix gave highly different results per injection, ranging from 24.9% to 48.3%. Therefore it has to be taken into account that certain variations in the measurements can always occur and that every sample should be measured more than once.

Table 22 Comparison of the average SC of CON (without the signal peptide) digested with immobilized trypsin and trypsin/LysC mix. SC = sequence coverage (%), MV = mean value (%), STDEV = standard deviation (%), a,b and c stand for different injections of the same sample technical replicate

	Immobilized trypsin			Trypsin/LysC mix		
	Tech. repl. 1	Tech. repl. 2	Tech. repl. 3	Tech. repl. 1	Tech. repl. 2	Tech. repl. 3
SC (%)	a) 34.3	a) 32.9	a) 26.1	a) 55.1	a) 24.9	a) 53.5
	b) 34.8	b) 35.1	b) 33.5	b) 49.7	b) 48.3	b) 60.5
	c) 34.1	c) 34.1	c) 33.8	c) 51.7	c) 37.0	c) 51.5
MV (%)	33.2			48.0		
STDEV (%)	2.7			10.7		

To detect eventually modified AA residues it was of interest to identify as many peptides as possible. In Figure 29 the total AA coverage for all injections is displayed. After digestion with immobilized trypsin a total SC of 313 AAs could be achieved (45.6%), while by trypsin/LysC mix digestion a total SC of 460 AAs could be accomplished (67.1%).

MASCOT scores for “Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution” (NCBInr entry: gi|83754919) ranged from 724 to 954 in results for protein digests using immobilized trypsin. MASCOT scores achieved with protein digests using trypsin/LysC mix ranged from 1000 to 1520 (with one spike of 602). This means that the identification of CON had a higher probability to be a true positive using trypsin/LysC mix for digestion. However CON was listed as identified in all cases as the threshold level for statistical significance was 63-65 ($p < 0.05$).

Immobilized trypsin:

APPKSVIRWCTISSPEE**K**KCNNLRDLTQQERISLTCVQ**K**ATYLDCI**K**AIANNEADAISLDGG
QVFEAGLAPY**K**LK**P**IAAEVYEHTEGSTTSYYAVAVV**K****K**GTEFTVNDLQG**K**TSCHTGLGRS
AGWNIPIGTLIHRGAIEWEGIESGSVEQAVA**K**FFSASCVPGATIEQ**K**LCRQCKGDPKTKCA
RNAPYSGYSGAFHCL**K**DG**K**GDVAFV**K**HTTVNENAPDQKDEYELLCLDGSRQPVDNYKTC
NWARVAAHAVVARDDNKVEDIWSFLSKAQSDFGVDT**K**SDFHLFGPPG**K**KDPVLKDLLFK
DSAIMLKRVPSLMDSQLYLGFEYYSAIQSMR**K**DQLTPSPRENRIQWCAVG**K**DE**K**SKCDR
WSVVSNGDVECTVVDETKDCIIKIMKGEADAVALDGGLVYTAGVCGLVPVMAERYDDESQ
CSKTDERPASYFAVAVAR**K**DSNVNWNNL**K**GKKSCHTAVGRTAGWVIPMGLIHNRTGTCN
FDEYFSEGCAPGSPPNSRLCQLCQGSGGIPPE**K**CVASSHEKYFGYTGALRCLVEKGDVA
FIQHSTVEENTGGKNKADWAKNLQMDDFELLCTDGRRANVMDYRECNLAEVPTHAVVVR
PE**K**ANKIRDLLERQEKRFGVNGSEKSKFMMFESQN**K**DLLFKDLTKCLFKVREGTTYKEFL
GDKFYTVISSL**K**TCNPSDILQMCSFLEG**K**

Total SC: 313 AA (45.6%)

Trypsin/LysC mix:

APPKSVIRWCTISSPEE**K**KCNNLRDLTQQERISLTCVQ**K**ATYLDCI**K**AIANNEADAISLDGG
QVFEAGLAPY**K**LK**P**IAAEVYEHTEGSTTSYYAVAVV**K****K**GTEFTVNDLQG**K**TSCHTGLGRS
AGWNIPIGTLIHRGAIEWEGIESGSVEQAVA**K**FFSASCVPGATIEQ**K**LCRQCKGDPKTKCA
RNAPYSGYSGAFHCL**K**DG**K**GDVAFV**K**HTTVNENAPDQ**K**DEYELLCLDGSRQPVDNYKTC
NWARVAAHAVVARDDN**K**VEDIWSFLS**K**AQSDFGVDT**K**SDFHLFGPPG**KK**DPVLKDLLFK
DSAIMLKRVPSLMDSQLYLGFEYYSAIQSMR**K**DQLTPSPRENRIQWCAVG**K**DE**K**SKCDR
WSVVSNGDVECTVVDETKDCIIKIM**K**GEADAVALDGGLVYTAGVCGLVPVMAERYDDESQ
CSKTDERPASYFAVAVAR**K**DSNVNWNNL**K**GKKSCHTAVGRTAGWVIPMGLIHNRTGTCN
FDEYFSEGCAPGSPPNSRLCQLCQGSGGIPPE**K**CVASSHEKYFGYTGALRCLVEKGDVA
FIQHSTVEENTGG**K**NKADWAKNLQMDDFELLCTDGRRANVMDYRECNLAEVPTHAVVVR
PE**K**ANKIRDLLERQEKRFGVNGSEKSKFMMFESQN**K**DLLF**K**DLT**K**CLFKVREGTTYKEFL
GD**K**FYTVISSL**K**TCNPSDILQMCSFLEG**K**

Total SC: 460 AA (67.1%)

Figure 29 Comparison of the total AA coverage of CON after 3 digestions with either immobilized trypsin or trypsin/LysC mix. Each digest was measured 3 times. Identified peptide sequences are highlighted in red. Identified lysine residues are displayed bold.

In respect to the goal of this master thesis the detection of as many Lys residues as possible was of interest, since the ZEN-CMO modification is most likely coupled to Lys. By using trypsin/LysC mix 32 Lys residues could be detected, compared to only 22 for immobilized trypsin. The Lys residues were detected quite frequently since the same Lys usually occurs in different peptides or the same peptide was detected in different charge states. The comparison

of the two different digestion methods showed that trypsin/LysC mix gave better results. A higher SC per injection and in total could be achieved and more Lys residues were covered.

At this point it is of interest to mention that it is very unlikely to achieve 100% SC for LC-MS/MS measurements. One major reason is the fact that not all peptides ionize with equal efficiency. Peptide size and charge are important factors for detection and fragmentation behavior influencing overall detectability in ESI-MS/MS studies.[93]

For correct interpretation of overall digestion performance, crucial differences in sample preparation should be acknowledged. The protocol for immobilized trypsin by Thermo Scientific recommends 0.10 – 0.25 ml of immobilized trypsin for the digestion of 1 mg protein. In this study 0.1 ml immobilized trypsin was used for 77 µg protein. This is due to the fact that the initial protein concentration was limited and that a minimum volume of agarose beads containing the immobilized trypsin has to be pipetted to prevent from picking up not enough beads from the stock solution. This however leads to a higher "beads-to-protein ratio" than recommended for the digestion. The exact amount of trypsin, which is immobilized on beads, is unknown.

Even though immobilization of trypsin should help remove trypsin after digestion and reduce autoproteolysis of the protease, autolytic peptides of the enzyme were regularly identified in LC-MS/MS measurements. Especially the triply charged ion $[M+3H]^{3+}$ at m/z 758.4 (K.SIVHPSYNSNTLNNDIMLIK.L) was observed in the sample in such a high intensity that it may be preferentially ionized. From this one may conclude that other ions resulting from peptides eluting between 46 and 51 min may have been suppressed and are therefore not observable (see Figure 30).

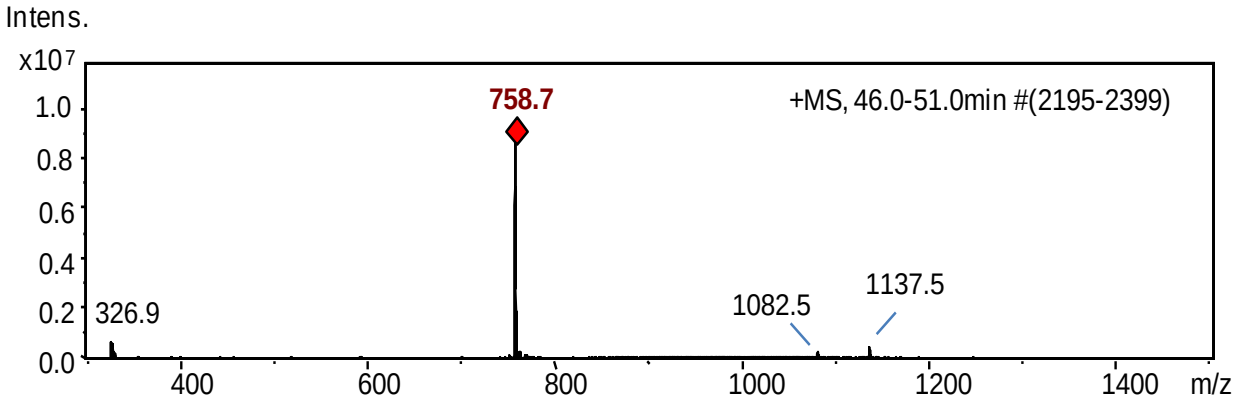


Figure 30 Mass profile of peptides eluting between 46.0 and 51.0 min after injecting 1 µL of CON digest using immobilized trypsin

This could lead to the conclusion that the digestion protocol for immobilized trypsin was not ideal and that better results might be achieved with a different digestion protocol, e.g. better adapted protein-to-protease ratio or shorter digestion times.

In Table 23 all identified peptides of bovine trypsin (immobilized trypsin) and porcine trypsin (contained in trypsin/LysC mix) are displayed. No proteolysis products of LysC could be identified.

Table 23 Identified autoproteolysis products of bovine and porcine trypsin

BOVINE TRYPSIN (Immobilized trypsin)	m/z	Charge	Sequence
	330.2	2+	K.SGIQVR.L
	453.8	2+	K.NKPGVYTK.V
	510.8	2+	K.APILSDSSCK.S
	556.3	2+	K.VCNYVSWIK.Q
	577.3	2+	K.SSGTSYPDVLK.C
	717.4	2+	K.LQGIVSWGSGCAQK.N
	721.7	3+	R.LGEDNINVVEGNEQFISASK.S
	758.4	3+	K.SIVHPSYNSNTLNNDIMLIK.L
	763.7	3+	K.SIVHPSYNSNTLNNDIMLIK.L + Ox. (M)
	1020.5	1+	K.APILSDSSCK.S
	1082.0	2+	R.LGEDNINVVEGNEQFISASK.S
	1097.5	2+	K.SAYPGQITSNMFCAGYLEGGK.D
	1137.1	2+	K.SIVHPSYNSNTLNNDIMLIK.L
PORCINE TRYPSIN (trypsin/LysC mix)	**m/z**	**Charge**	**Sequence**
	421.8	2+	R.VATVSLPR.S
	523.3	2+	K.LSSPATLNSR.V
	737.7	3+	R.LGEHNIDVLEGNEQFINAAK.I
	761.7	3+	K.IITHPNFNGNTLDNDIMLIK.L

Another difference in the two digestion protocols is the resuspension buffer for the proteases:

Immobilized trypsin is washed with the digestion buffer (40 mM NH_4HCO_3 (pH 8.5), 1.2 M urea, 0.4 M thiourea) before use.

Trypsin/LysC mix is however dissolved in 50 mM acetic acid (resuspension buffer) for storage. Concentration of the trypsin/LysC mix solution used in this experiment was 40 ng/µl. For this, 77 µl 50 mM acetic acid, containing the enzyme mix, were filled up to 500 µl with digestion buffer. The enzyme is stored in acetic acid to inactivate the enzyme and prevent autoproteolysis during storage. The combination of resuspension and digestion buffer results in an overall decrease of the pH of the digestion buffer. Since the optimum pH value ranges from 8.0 to 9.0 for trypsin and from 7.5 to 8.5 for LysC[94] this may not have too much of a negative effect on protein digestion, especially as the manufacturers of both proteases recommend pH 8.0 for optimum digestion.

Also the trypsin origin is different for the two proteases. Immobilized trypsin is bovine trypsin, while the trypsin in the trypsin/LysC mix stems from pigs (porcine trypsin), both differing in 18% of their amino acid sequence.

Walmsley et al.[95] performed a study using six different trypsins for digestion. In their work bovine trypsin produced peptides with more missed cleavages than porcine trypsin. The different enzyme sequences have different binding affinities to the substrate proteins, depending on the sequence properties of the substrate. Additionally, protease specificity is influenced by its substrate binding pocket and how well substrate and enzyme surfaces fit together.[95] For this work it is of notice, that it is not known whether bovine or porcine trypsin has a higher binding affinity to CON.

In summary trypsin/LysC mix might have given better results due to one or all of these factors. For this work high SC was important to identify possible modification sites. Therefore trypsin/LysC mix was used for further experiments.

3.3 Determination of modification sites on a ZEN-CMO-CON conjugate

To gain more information on the conjugation reaction, the positions of ZEN-CMO modifications on the proteins were of interest. Therefore the modified AA residues had to be determined. In order to achieve this, the different ZEN-CMO-CON conjugates were digested with trypsin/LysC mix, desalted using C_{18} spin columns and the MS/MS spectra were investigated using software packages as DataAnalysis v3.2 and BioTools v3.2 (Bruker Daltonics). Basic fragmentation pattern of ZEN-CMO modified peptides and ZEN-CMO itself were previously studied by MALDI-TOF/TOF-MS in the master thesis of C. Stephan.[91] First it had to be confirmed that this fragmentation pattern is similar to low energy CID fragmentation in QIT.

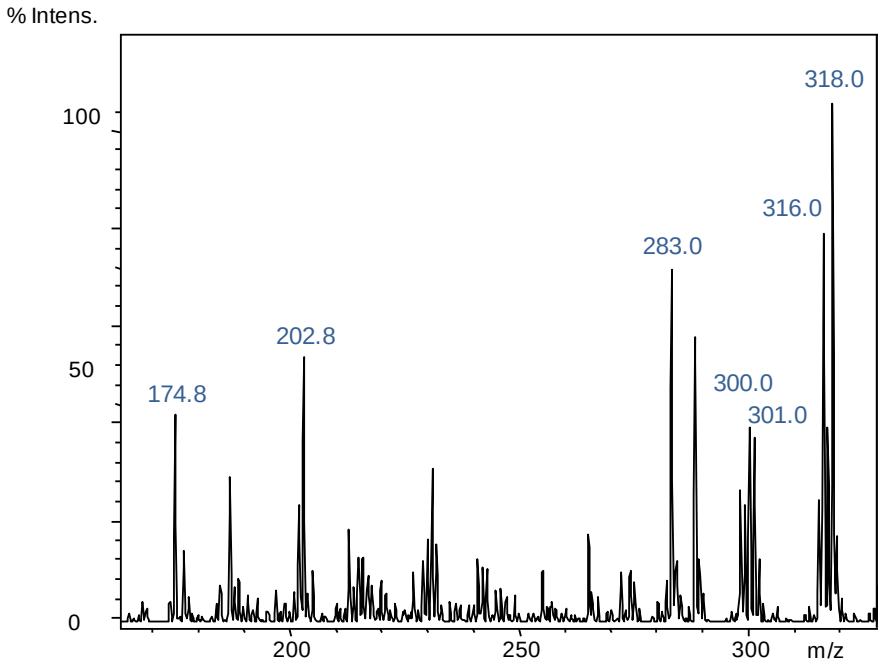


Figure 31 Fragment ions resulting from low energy CID experiments of a ZEN-CMO modified peptide K.KCNNLR.D 1: ZEN-CMO (K), 2:CAM (C) (m/z 589.3) generated in a 3D QIT. The displayed peak pattern in MS/MS spectra was taken as verification that the spectrum corresponded to a modified peptide (labeled peaks are characteristic for the ZEN-CMO fragmentation)

MS/MS spectra were manually investigated for possible ZEN-CMO reporter ions. The modified peptide MS/MS spectra had a very distinctive recurring peak pattern in the lower mass range (see Figure 31). The most intense ions of the pattern are at m/z 174.8, m/z 202.8, m/z 283.0, m/z 300.0, m/z 301.0, m/z 316.0 and m/z 318.0. The mass accuracy for the measured ions is m/z ± 0.1.

The fragment ions at m/z 203 and m/z 316 were also used in the work of C.Stephan to verify ZEN-CMO modification.[91] All fragment ions (except m/z 283) are also observable in MALDI-TOF-MS/MS spectra. [91]

Yet, QIT characteristics do not allow detecting all reporter ions for any given peptide. With increasing m/z values the ions m/z 174.8, m/z 202.8 and m/z 283.0 can no longer be trapped (see chapter 1.6.2 for more detailed information) and can therefore not be seen in the mass spectrum.

In almost all MS/MS spectra, m/z values corresponding to a peptide with one CMO molecule were detected, which means that the bond between ZEN and CMO molecule is easily broken during CID. This has also been observed previously in the work of C. Stephan.[91] Peaks with only

the CMO part attached were further indication that a modified peptide was identified. If only the CMO molecule is attached to the peptide, monoisotopic mass increases by 58 Da compared to the unmodified peptide. For spectra of the modified peptides see appendix.

In Table 24 all identified modified peptides are shown along with the detected reporter ions. In total 33 ions could be detected that correspond to 32 different peptides. Most peptides were found to carry one ZEN-CMO modification, but in two cases the peptide even carried 2 modifications.

Table 24 m/z value, sequence and charge of ZEN-CMO modified peptides and associated reporter ions

			Reporter ions m/z						
m/z	Sequence	Mass (Da)	175	203	283	300	301	316	318
463.6^{3+}	**K.KSCHTAVGR.T** 1: ZEN-CMO (K), 3: CAM (C)	1387.7	x	x	x	x	x	x	x
467.2^{2+}	**R.QEKR.F** 3: ZEN-CMO (K)	932.5	x	x	x	x	x	x	x
487.8^{2+}	**K.ANKIR.D** 3: ZEN-CMO (K)	973.5	x	x	x	x	x	x	x
504.7^{2+}	**K.TKCAR.N** 2: ZEN-CMO (K), 3: CAM (C)	1007.5	x	x	x	x	x	x	x
519.7^{2+}	**K.SKCDR.W** 2: ZEN-CMO (K), 3: CAM (C)	1037.4	x	x	x	x	x	x	x
536.8^{2+}	**K.KDPVLK.D** 1: ZEN-CMO (K)	1071.6	x	x	x	x	x	x	x
568.3^{3+}	**K.SDFHLFGPPGKK.D** 11: ZEN-CMO (K)	1701.8	x	x	x	x	x	x	x
589.3^{2+}	**K.KCNNLR.D** 1: ZEN-CMO (K), 2:CAM (C)	1176.6	x	x	x	x	x	x	x
598.3^{2+}	**K.CLFKVR.E** 1:CAM (C), 4: ZEN-CMO (K)	1194.6	x	x	x	x	x	x	x
603.3^{2+}	**K.NKADWAK.N** 2: ZEN-CMO (K)	1204.6	x	x	x	x	x	x	x
603.3^{2+}	**R.QCKGDPK.T** 2: CAM (C), 3: ZEN-CMO (K)	1204.5	x	x	x	x	x	x	x
620.8^{2+}	**-.APPKSVIR.W** 4: ZEN-CMO (K)	1239.7	x	x	x	x	x	x	x

630.6^{3+}	**R.KDSNVNWNNLKGK.K** 11: ZEN-CMO (K)	1888.9		x	x	x	x	x	x
653.8^{2+}	**K.DSAIMLKR.V** 7: ZEN-CMO (K)	1305.7		x	x	x	x	x	x
699.3^{2+}	**K.DLTKCLFK.V** 4: ZEN-CMO (K), 5: CAM (C)	1396.7		x	x	x	x	x	x
704.8^{2+}	**K.DGKGDVAFVK.H** 3: ZEN-CMO (K)	1407.7		x	x	x	x	x	x
707.9^{2+}	**R.KDQLTPSPR.E** 1: ZEN-CMO (K)	1413.7		x	x	x	x	x	x
713.3^{2+}	**R.FGVNGSEKSK.F** 8: ZEN-CMO (K)	1424.7		x	x	x	x	x	x
752.4^{3+}	**K.SDFHLFGPPGKKDPVLK.D** 11: ZEN-CMO (K)	2254.2			x	x	x	x	x
852.9^{2+}	**R.KDSNVNWNNLK.G** 1: ZEN-CMO (K)	1703.8			x	x	x	x	x
853.9^{2+}	**R.IQWCAVGKDEK.S** 4: CAM (C), 8: ZEN-CMO (K)	1705.8			x	x	x	x	x
869.4^{2+}	**R.WCTISSPEEKK.C** 2: CAM (C), 10: ZEN-CMO (K)	1736.8			x	x	x	x	x
875.4^{2+}	**K.SKFMMFESQNK.D** 2: ZEN-CMO (K)	1748.8			x	x	x	x	x
881.4^{2+}	**K.DSNVNWNNLKGK.K** 10: ZEN-CMO (K)	1760.8			x	x	x	x	x
889.9^{2+}	**K.GDPKTKCAR.N** 4: ZEN-CMO (K), 6: ZEN-CMO (K), 7: CAM (C)	1777.8			x	x	x	x	x
904.4^{2+}	**R.QCKGDPKTK.C** 2: CAM (C), 3: ZEN-CMO (K), 7: ZEN-CMO (K)	1806.8			x	x	x	x	x
905.4^{2+}	**K.KGTEFTVNDLQGK.T** 1: ZEN-CMO (K)	1808.9			x	x	x	x	x
933.8^{3+}	**R.EGTTYKEFLGDKFYTVISSLK.T** 12: ZEN-CMO (K)	2798.4			x	x	x	x	x
945.5^{2+}	**R.KDSNVNWNNLKGK.K** 11: ZEN-CMO (K)	1888.9			x	x	x	x	x
954.5^{2+}	**R.SAGWNIPIGTLLHR.G** 1: ZEN-CMO (S)	1907.0			x	x	x	x	x

1013.0^{2+}	**R.QPVDNYKTCNWAR.V** 7: ZEN-CMO (K), 9:CAM (C)	2023.9			x	x	x	x	x
1060.5^{2+}	**K.EFLGDKFYTVISSLK.T** 6: ZEN-CMO (K)	2119.1				x	x	x	x
1076.0^{2+}	**K.FMMFESQNKDLLFK.D** 9: ZEN-CMO (K)	2150.0				x	x	x	x

In Figure 32 all modified AA residues are shown. In total 28 modification sites could be detected, 27 Lys and even one Ser. Peptides were usually identified as doubly or triply charged.

```
        10         20         30         40         50
APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
        60         70         80         90         100
NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
       110        120        130        140        150
GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLLHRGAIEW EGIESGSVEQ
       160        170        180        190        200
AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
       210        220        230        240        250
GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
       260        270        280        290        300
AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
       310        320        330        340        350
KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
       360        370        380        390        400
GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
       410        420        430        440        450
TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
       460        470        480        490        500
KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
       510        520        530        540        550
LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
       560        570        580        590        600
GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
       610        620        630        640        650
ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
       660        670        680

TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Figure 32 Protein sequence of CON highlighting AA in red that were identified to be modified. 27 out of 59 Lys were identified to be actually modified plus 1 Ser. Underlined is the sequence of the protein covered by the tryptic digestion.

As described earlier, Lys is the most likely modification site, yet also Arg, Ser or Thr may be affected. From the identified modified peptides can be positively concluded, that the NH_2-group of Lys is the preferred residue for coupling. Almost all modifications could be identified on Lys. No modified Arg or Thr could be detected. However one modified Ser was identified (see Figure 33) in the measurements of sample B4, a sample modified with cr 1:50 and overnight activation. It seems therefore that at a high molar ratio and a long activation time coupling is also possible for Ser. At a low cr however Lys residues seem to be preferred. MS/MS spectra for modified peptides are attached in the appendix.

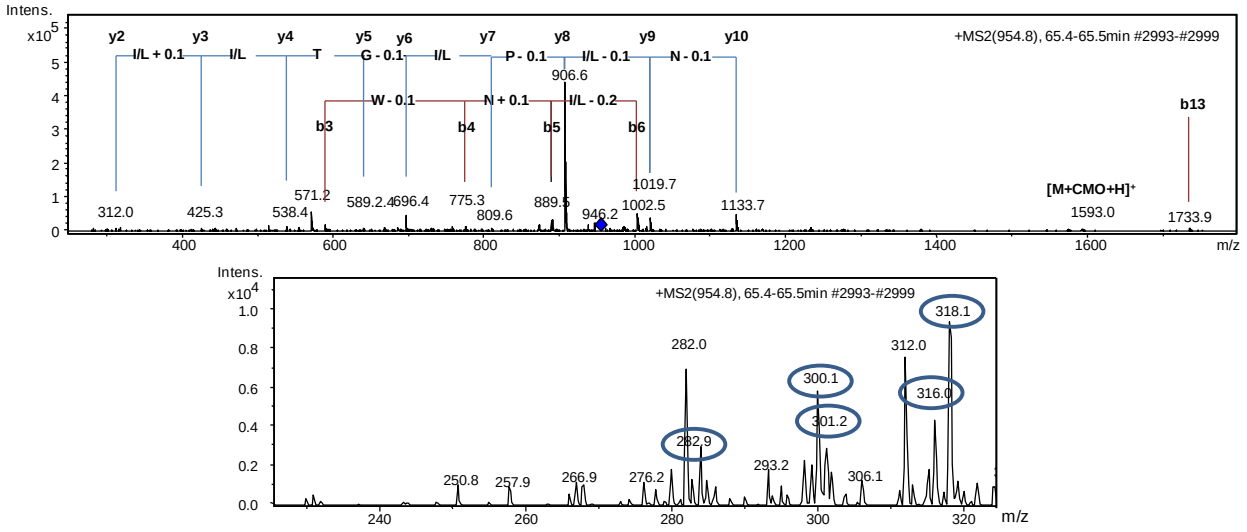


Figure 33 MS/MS spectrum of the ZEN-CMO modified peptide R.SAGWNIPIGTLLHR.G (m/z 954.5^{2+}). Ser is the modified AA residue. y – ion series is displayed in blue, b – ion series is displayed in red. On the bottom the identified reporter ions are shown.

It is important to clarify that QIT data are not preferred for quantitative analysis because of the limited dynamic range and the fact that MS data per sé are not quantifiable. So it cannot be stated to which degree an AA residue is modified. Taking previous MALDI-linTOF-MS results into account, it is known that a protein treated with cr 1:50 and over night activation has an average overall modification of 13-17 attached ZEN-CMO molecules (depending on conjugation conditions and sample measurement – internal or external calibration).[91] And nLC-nESI-MS/MS data show that these modifications are attached not to just one AA residue, but the modifications are widely spread over the whole protein sequence, showing that there is not one preferential modification site.

It was interesting to observe that all peptides with a modified Lys residue contain a missed cleavage at the modified AA residue. This leads to the conclusion that ZEN-CMO modification

poses a steric hindrance for the proteases and prevents peptide cleavage at the modified Lys during protein digestion. Therefore different peptides might be formed during protein digestion depending on present ZEN-CMO modification.

Further it was of interest to identify reporter ions, which were used for verification that a modified peptide was identified. Since the same (see Figure 31) peaks occurred in all spectra of modified peptides it was expected that the fragment ions stem from the ZEN-CMO modification.

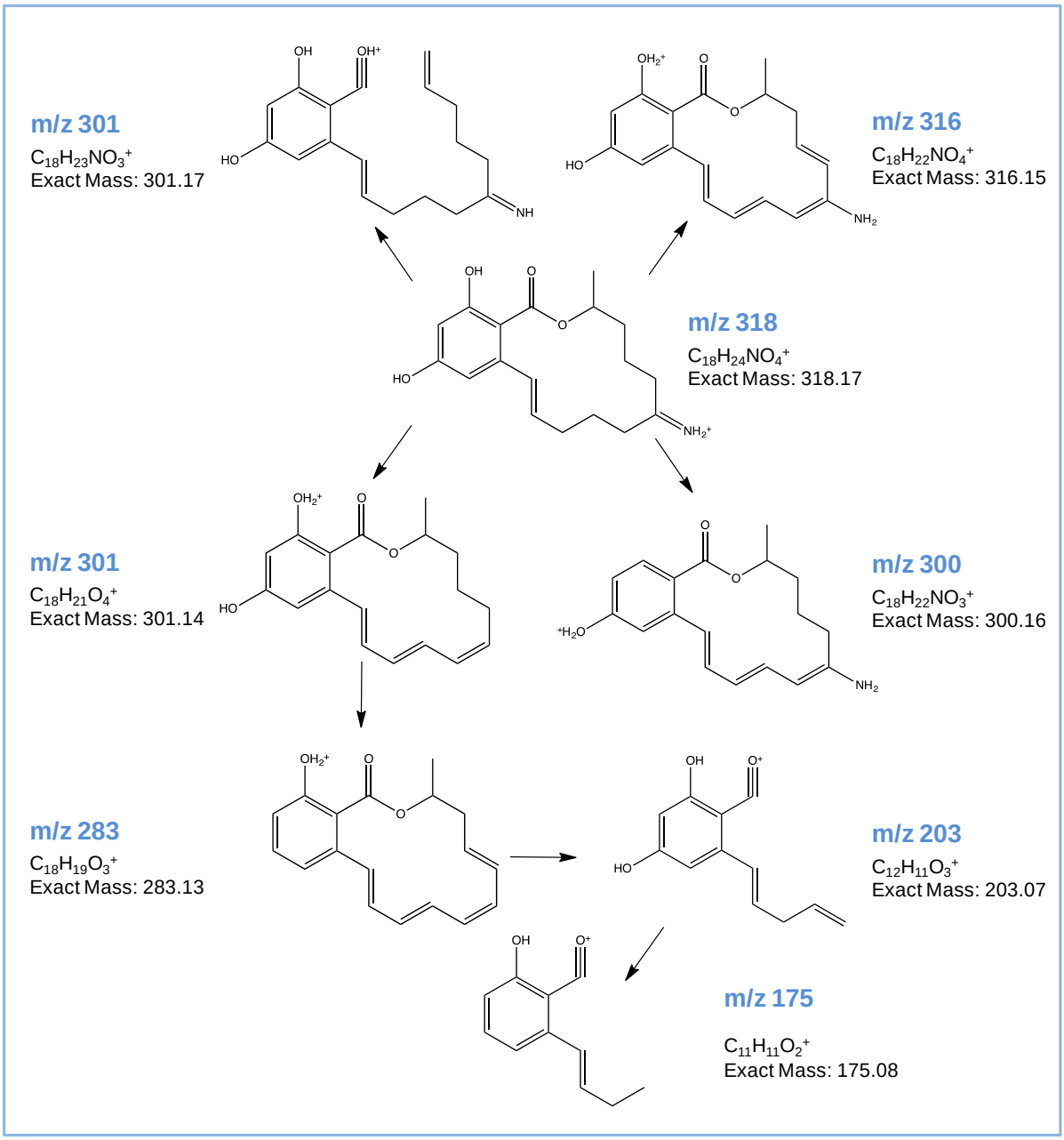


Figure 34 Putative structures of ZEN-CMO fragment ions. Monoisotopic masses are corresponding to the m/z value of the reporter ions

In Figure 34 putative structures for the ZEN-CMO fragment ions are given, which mass could be assigned to the identified peaks according to their m/z. For further information on fragmentation see chapter 3.5.1.

Further it was of interest to gain information on where the modified AA residues are located. Figure 35 shows the structure of CON (PDB entry: 1AIV) in the form of a “Tube worm” model. The protein was edited in Cn3D. Identified modification sites are highlighted in yellow and displayed as a “ball and stick” model.

Figure 35 CON displayed as a "Tube worm" model. Identified possible ZEN-CMO modification sites are highlighted in yellow. (Source: NCBI, PDB entry: 1AIV)

The 3D display of the protein showed once again that ZEN-CMO modifications are widely spread over the whole protein. It also seems as if the modified AA residues are located on the surface of the protein. This makes sense, since there they are easily accessible for coupling.

3.4 Influence of the modification on retention time

ZEN is known to be a highly hydrophobic substance.[4] Therefore it was expected that a modified peptide would be more hydrophobic than the corresponding unmodified one. Since RPC, which is based on hydrophobic interactions of the analytes with the stationary and mobile phase (see chapter 1.5.1), was used to separate the peptides, it was of interest to determine whether ZEN-CMO modification would change a peptide´s RT.

The problem at this point was that in most cases the corresponding unmodified peptide could not be detected in the nLC-nESI-QIT measurement. In some cases the unmodified peptides were only identified in measurements with different gradients, but those were disadvantageous for the modified analogue. Therefore RTs could simply not be compared.

So, for theoretical considerations the RTs of the unmodified peptides were calculated with TheorChromo 1.0.[92] In this software chromatographic parameters, like column length and flow rate are inserted and theoretical RTs are calculated for each submitted AA sequence. Most modified AA residues could be determined in measurements using gradient B (see 2.5.1). The chromatographic parameters were adapted to fit this gradient (see chapter 2.6.2).

It is known that the calculation of RTs for peptides is very difficult and usually not correct, but a very good starting point for further investigations. After theoretical RTs were determined, they were compared to RTs of the unmodified peptides, which could actually be identified in nLC-nESI-QIT measurements, to verify if the values are similar. Since the determined values only differed by 1-2 min (except in the case of the sequence K.FMMFESQNKDLLFK.D showing an 8 min delay) it was confirmed that the theoretical values could be used to estimate the RTs of the unmodified peptides. In cases where the RT for an unmodified peptide could be correlated to a modified peptide, RT shifts were calculated directly, without using the theoretical value.

RTs for the modified peptides K.SDFHLFGPPGKK.D (11: ZEN-CMO) and K.SDFHLFGPPGKKDPVLK.D (11: ZEN-CMO) were not included in the calculation since these modification sites were identified in measurements using Gradient A and the results are therefore not comparable. For information on the different gradients see chapter 2.5.1. Also peptides R.QCKGDPKTK.C and K.GDPKTKCAR.N were excluded as well, since these peptides carry two ZEN-CMO modifications.

Differences in RTs are shown in Table 25. Further the percentage of solvent B at the moment of elution is given. For this calculation a delay time of 28.0 min (according to “TheorChromo”) was

used. Also the GRAVY (grand average of hydropathy) score is presented for each peptide. The GRAVY score is calculated by summing up the hydropathy values of all AAs and dividing that number by the peptide length. The GRAVY score was calculated via http://www.gravy-calculator.de/.[96] Carbamidomethylation of the peptide could not be taken into account to calculate the GRAVY score.

Table 25 Evaluation of the influence of ZEN-CMO modification on a peptide´s RT on nLC. Theoretical RT of unmod. peptides was determined using "TheorChromo". Observed RT of unmod. peptides means the mean value of RTs determined from nLC-nESI-QIT measurements. RT difference between the modified and the unmodified peptide is displayed as RT shift. Modifications of AAs are described after the sequence (position: modification type (AA), CAM=Carbamidomethylation, ZEN-CMO = ZEN-CMO modification)

Sequence	Theor. RT (min)	Obs. RT (min)	mod. Peptide RT (min)	RT Shift (min)	GRAVY Score (No ZEN-CMO, no CAM)	Theor. ACN at elution of unmod. peptide (%)	Theor. ACN at elution of mod. peptide (%)
1 ZEN-CMO-modification							
R.QEKR.F 3: ZEN-CMO (K)	30.0	-	53.2	+ 23.2	-3.85	5.0	18.7
K.SKCDR.W 2: ZEN-CMO (K), 3: CAM (C)	30.0	-	53.9	+ 23.9	-2.04	5.0	19.3
K.TKCAR.N 2: ZEN-CMO (K), 3: CAM (C)	30.1	-	52.7	+ 22.6	-0.96	5.0	18.2
R.QCKGDPK.T 2: CAM (C), 3: ZEN-CMO (K)	30.2	-	53.0	+ 22.8	-2.04	5.0	18.5
K.ANKIR.D 3: ZEN-CMO (K)	30.6	-	54.1	+ 23.5	-1.12	5.0	19.5
K.KSCHTAVGR.T 1: ZEN-CMO (K), 3: CAM (C)	31.0	-	48.7	+ 17.7	-0.56	5.0	14.6
K.KCNNLR.D 1: ZEN-CMO (K), 2: CAM (C)	31.2	-	53.4	+ 22.2	-1.52	5.0	18.9
R.FGVNGSEKSK.F 8: ZEN-CMO (K)	31.8	-	55.6	+ 23.8	-1.02	5.0	20.8
R.KDQLTPSPR.E 1: ZEN-CMO (K)	32.6	-	55.9	+ 23.3	-1.81	5.0	21.1
-.APPKSVIR.W 4: ZEN-CMO (K)	33.7	-	56.0	+ 22.3	-0.24	5.0	21.2

K.NKADWAK.N 2: ZEN-CMO (K)	34.0	-	58.5	+ 24.5	-1.73	5.0	23.4
K.KDPVLK.D 1: ZEN-CMO (K)	34.2	-	56.7	+ 22.5	-0.82	5.0	21.8
R.WCTISSPEEKK.C 2: CAM (C), 10: ZEN-CMO (K)	34.8	-	57.2	+ 22.4	-1.15	5.0	22.3
K.CLFKVR.E 1:CAM, 4: ZEN-CMO (K)	36.0	-	60.1	+ 24.1	0.82	5.0	24.9
R.KDSNVNWNNLKGK.K 11: ZEN-CMO (K)	36.0	-	56.2	+ 20.2	-1.79	5.0	21.4
K.DSNVNWNNLKGK.K 10: ZEN-CMO (K)	36.4	-	61.5	+ 25.1	-1.62	5.0	26.2
R.QPVDNYKTCNWAR.V 7: ZEN-CMO (K), 9:CAM (C)	36.8	-	58.4	+ 21.6	-1.42	5.0	23.4
R.IQWCAVGKDEK.S 4: CAM (C), 8: ZEN-CMO (K)	37.1	39.3	58.6	+ 19.3	-0.60	5.0	23.6
K.DSAIMLKR.V 7: ZEN-CMO (K)	39.0	-	60.9	+ 21.9	-0.1	5.9	25.6
R.KDSNVNWNNLK.G 1: ZEN-CMO (K)	40.6	-	57.6	+ 17.0	-1.73	7.4	22.6
K.DLTKCLFK.V 4: ZEN-CMO (K), 5: CAM (C)	41.2	-	64.4	+ 23.2	0.11	7.9	28.8
K.DGKGDVAFVK.H 3: ZEN-CMO (K)	41.4	-	61.2	+ 19.8	-0.26	8.1	25.9
K.KGTEFTVNDLQGK.T 1: ZEN-CMO (K)	41.5	40.6	58.9	+ 18.3	-1.015	8.1	23.8
K.SKFMMFESQNK.D 2: ZEN-CMO (K)	44.4	-	62.5	+ 18.1	-0.95	10.8	27.1
R.SAGWNIPIGTLLHR.G 1: ZEN-CMO (S)	51.8	51.3	65.4	+ 14.1	0.17	17.4	29.6
R.EGTTYKEFLGDKFYTV ISSLK.T 12: ZEN-CMO (K)	55.5	-	68.0	+ 12.5	-0.35	20.8	32.0
K.EFLGDKFYTVISSLK.T 6: ZEN-CMO (K)	55.8	55.9	71.1	+ 15.2	0.21	21.0	34.8
K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K)	59.4	51.4	65.9	+ 14.5	-0.20	24.3	30.1

2 ZEN-CMO-modifications							
R.QCKGDPKTK.C 2: CAM (C), 3: ZEN-CMO (K), 7: ZEN-CMO (K)	30.1	-	64.6	+ 34.5	-2.10	5.0	28.9
K.GDPKTKCAR.N 4: ZEN-CMO (K), 6: ZEN-CMO (K), 7: CAM (C)	30.3	-	65.5	+ 35.2	-1.58	5.0	29.8

From these results can clearly be concluded that ZEN-CMO modification significantly increases a peptide´s RT. This confirms that ZEN-CMO is a hydrophobic substance and that therefore the modified peptide is more hydrophobic than the unmodified peptide.

On average one ZEN-CMO modification leads to an increase in RT of about 20.7 ± 3.5 minutes. RT is of course depending on the gradient used for elution. The increase in RT was not equal for all peptides. The highest delay in RT was observed for peptide K.NKADWAK.N (24.5 minutes). RT for peptide R.EGTTYKEFLGDKFYTVISSLK.T on the other hand was only delayed by 12.5 minutes. The RT shifts are displayed in Figure 36. The peptides are sorted by peptide length.

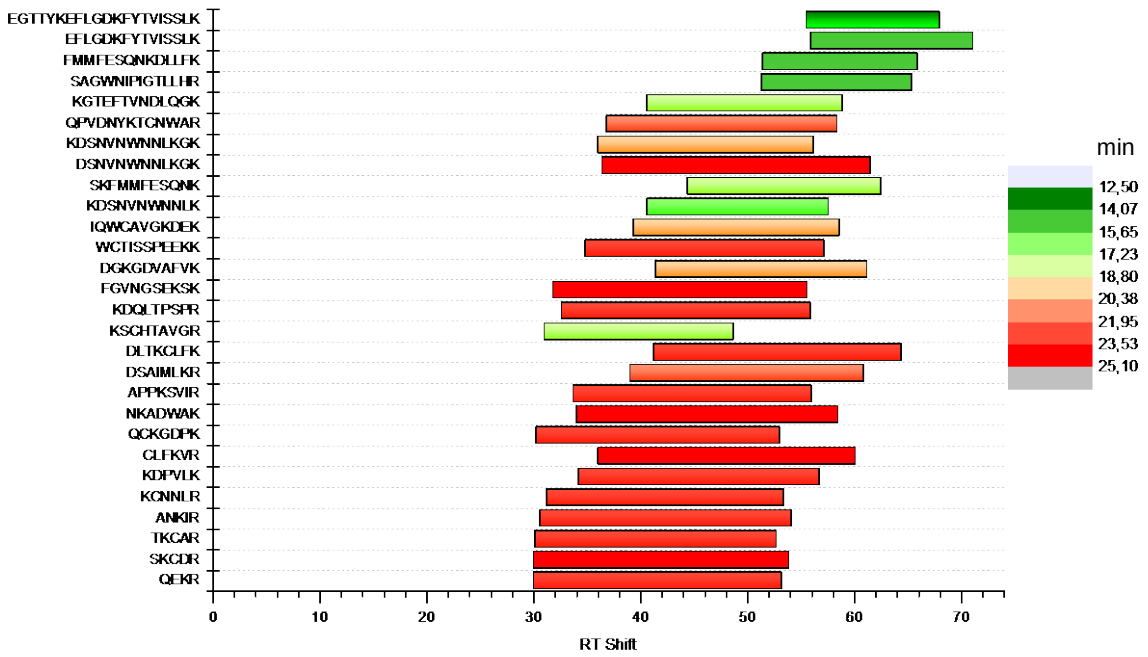


Figure 36 Peptide sequences sorted by peptide length. The RT differences between unmodified and ZEN-CMO modified peptide on the nLC are displayed as bars. Theoretical RTs were used if the peptide could not be detected

According to the figure ZEN-CMO modification had a higher influence on the RT shift of small peptides. Actual RTs were however only observed for peptides with a higher RT. It is possible that the theoretical gradient by “TheorChromo” does not fit well to the RTs of the smaller peptides, which can also lead to the discrepancy in the RT shift between smaller and larger peptides.

The increase of hydrophobicity, if a peptide is ZEN-CMO modified, is a positive effect for RPC, since the peptide will be better retained and is unlikely to be part of the flow through. However some of the peptides may get so hydrophobic that they get lost, because of irreversible binding, during the desalting process on the C_{18} material.

Further a high RT is an additional parameter to determine peptide modification.

The influence of the ZEN-CMO modification on RT was also investigated for UPLC measurements to examine whether the different LC system would have a different effect on the RT shift between unmodified and ZEN-CMO modified peptide.

In UPLC measurements 4 unmodified peptides were identified. Based on the RTs of these peptides theoretical RTs for other unmodified peptides were estimated by using “TheorChromo”. By using the correct chromatographic parameters, the predicted RTs did not match the observed ones. Therefore the chromatographic parameters inserted in “TheorChromo” were adapted that the theoretical RTs fit the observed ones as close as possible. The chromatographic parameters are shown in Table 20. The theoretical and the observed RT for the early eluting peptide R.KDQLTPSPR.E 1: ZEN-CMO (K) are exactly the same. They also fit closely for the late eluting peptide R.SAGWNIPIGTLLHR.G 1: ZEN-CMO (S). In between however the difference between theoretical and observed RT is further off (2.8 min for peptide R.IQWCAVGKDEK.S 4: CAM (C), 8: ZEN-CMO (K)). See Table 26 for further information. For the determination of the amount of solvent B at the point of elution a delay time of 2 min was used.

Table 26 Evaluation of the influence of ZEN-CMO modification on a peptide´s RT on UPLC. Theoretical RT of unmod. peptides was determined using "TheorChromo". RT difference between the modified and the unmodified peptide is displayed as RT shift. Modifications of AAs are described after the sequence (position: modification type (AA), CAM=Carbamidomethylation, ZEN-CMO = ZEN-CMO Modification)

Sequence	Theor. RT (min)	Obs. RT (min)	mod. Peptide RT (min)	RT Shift (min)	GRAVY Score (No ZEN-CMO, no CAM)	Theor. ACN at elution of unmod. peptide (%)	Theor. ACN at elution of mod. peptide (%)
1 ZEN-CMO-modification							
R.QEKR.F 3: ZEN-CMO (K)	2.5	-	20.5	18.0	-3.85	5.5	23.5
K.SKCDR.W 2: ZEN-CMO (K), 3: CAM (C)	2.5	-	20.6	18.1	-2.04	5.5	23.6
K.TKCAR.N 2: ZEN-CMO (K), 3: CAM (C)	2.5	-	20.7	18.2	-0.96	5.5	23.7
R.QCKGDPK.T 2: CAM (C), 3: ZEN-CMO (K)	2.6	-	20.8	18.2	-2.04	5.6	23.8
K.ANKIR.D 3: ZEN-CMO (K)	2.7	-	23.5	20.8	-1.12	5.7	26.5
K.KSCHTAVGR.T 1: ZEN-CMO (K), 3: CAM (C)	2.9	-	19.5	16.6	-0.56	5.9	22.5
K.KCNNLR.D 1: ZEN-CMO (K), 2:CAM (C)	2.9	-	22.1	19.2	-1.52	5.9	25.2
R.FGVNGSEKSK.F 8: ZEN-CMO (K)	3.2	-	24.0	20.8	-1.02	6.2	27.0
R.KDQLTPSPR.E 1: ZEN-CMO (K)	3.6	3.6	23.7	20.1	-1.81	6.6	26.7
-.APPKSVIR.W 4: ZEN-CMO (K)	4.0	-	26.3	22.3	-0.24	7.0	29.3
K.NKADWAK.N 2: ZEN-CMO (K)	4.2	-	26.1	21.9	-1.73	7.2	29.1
K.KDPVLK.D 1: ZEN-CMO (K)	4.3	-	25.8	21.5	-0.82	7.3	28.8
R.WCTISSPEEKK.C 2: CAM (C), 10: ZEN-CMO (K)	5.1	-	25.9	20.8	-1.15	8.1	28.9

K.CLFKVR.E 1:CAM, 4: ZEN-CMO (K)	5.3	-	29.7	24.4	0.82	8.3	32.7
R.KDSNVNWNNLKGK.K 11: ZEN-CMO (K)	6.2	-	26.6	20.4	-1.79	9.2	29.6
K.DSNVNWNNLKGK.K 10: ZEN-CMO (K)	6.5	-	27.9	21.4	-1.62	9.5	30.9
R.QPVDNYKTCNWAR.V 7: ZEN-CMO (K), 9:CAM (C)	6.6	-	26.9	20.3	-1.42	9.6	29.9
R.IQWCAVGKDEK.S 4: CAM (C), 8: ZEN-CMO (K)	6.9	9.7	27.6	17.9	-0.60	9.9	30.6
K.DSAIMLKR.V 7: ZEN-CMO (K)	7.7	-	28.9	21.2	-0.1	10.7	31.9
R.KDSNVNWNNLK.G 1: ZEN-CMO (K)	9.5	-	25.8	16.3	-1.73	12.5	28.8
K.DLTKCLFK.V 4: ZEN-CMO (K), 5: CAM (C)	9.2	-	31.5	22.3	0.11	12.2	34.5
K.DGKGDVAFVK.H 3: ZEN-CMO (K)	9.5	-	27.6	18.1	-0.26	12.5	30.5
K.KGTEFTVNDLQGK.T 1: ZEN-CMO (K)	11.4	11.0	26.6	15.6	-1.015	14.4	29.6
K.SKFMMFESQNK.D 2: ZEN-CMO (K)	13.8	-	32.0	18.2	-0.95	16.8	35.0
R.SAGWNIPIGTLLHR.G 1: ZEN-CMO (S)	22.7	22.9	35.6	12.7	0.17	25.7	38.6
R.EGTTYKEFLGDKFYTV ISSLK.T 12: ZEN-CMO (K)	27.5	-	40.6	13.1	-0.35	30.5	43.6
K.EFLGDKFYTVISSLK.T 6: ZEN-CMO (K)	27.3	-	41.6	14.3	0.21	30.3	44.6
K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K)	29.8	-	36.1	6.3	-0.20	32.8	39.1
2 ZEN-CMO-modifications							
R.QCKGDPKTK.C 2: CAM (C), 3: ZEN-CMO (K), 7: ZEN-CMO (K)	2.5	-	33.7	31.2	-2.10	5.5	36.7
K.GDPKTKCAR.N 4: ZEN-CMO (K), 6: ZEN-CMO (K), 7: CAM (C)	2.5	-	34.4	31.9	-1.58	5.5	37.4

According to UPLC measurements 1 ZEN-CMO modification leads on average to a RT increase of 18.1 ± 3.7 min, which is 2.6 min less than in nLC measurements. The difference in RT shift may occur due to the different gradients and the different LC systems.

Comparison of the RTs of unmodified and ZEN-CMO modified peptides showed again that ZEN-CMO modification leads to an increase in RT. The highest shift was observed for peptide K.CLFKVR.E 1: CAM, 4: ZEN-CMO (K) with 24.4 min, while the smallest was observed for peptide K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K) (6.3 min).

In Figure 37 the RT shift on the UPLC is shown. The difference of the influence of the ZEN-CMO modification on RT between smaller and larger peptides is not as clearly observable as on the nLC. However it still seems that the ZEN-CMO modification has the least influence on the RT of bigger peptides.

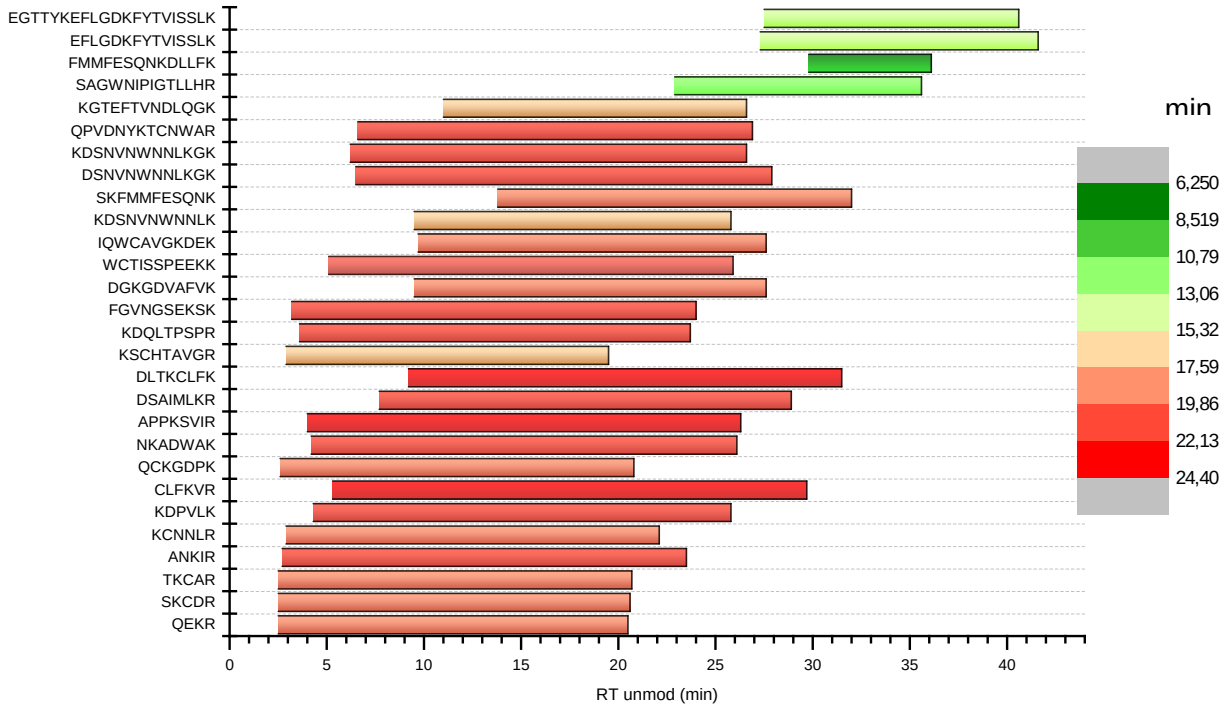


Figure 37 Peptide sequences sorted by peptide length. The RT differences between unmodified and ZEN-CMO modified peptide on the UPLC are displayed as bars. Theoretical RTs were used if the peptide could not be detected

Finally the RT difference of the ZEN-CMO modified peptides between nLC and UPLC were compared.

Table 27 Comparison of modified peptides RT in nLC compared to UPLC measurements

Sequence	RT nLC	RT UPLC	Difference
R.QEKR.F 3: ZEN-CMO (K)	53.2	20.5	- 32.7
K.SKCDR.W 2: ZEN-CMO (K), 3: CAM (C)	53.9	20.6	- 33.3
K.TKCAR.N 2: ZEN-CMO (K), 3: CAM (C)	52.7	20.7	- 32.0
R.QCKGDPK.T 2: CAM (C), 3: ZEN-CMO (K)	53.0	20.8	- 32.2
K.ANKIR.D 3: ZEN-CMO (K)	54.1	23.5	- 30.6
K.KSCHTAVGR.T 1: ZEN-CMO (K), 3: CAM (C)	48.7	19.5	- 29.2
K.KCNNLR.D 1: ZEN-CMO (K), 2:CAM (C)	53.4	22.1	- 31.3
R.FGVNGSEKSK.F 8: ZEN-CMO (K)	55.6	24.0	- 31.6
R.KDQLTPSPR.E 1: ZEN-CMO (K)	55.9	23.7	- 32.2
-.APPKSVIR.W 4: ZEN-CMO (K)	56.0	26.3	- 29.7
K.NKADWAK.N 2: ZEN-CMO (K)	58.5	26.1	- 32.4
K.KDPVLK.D 1: ZEN-CMO (K)	56.7	25.8	- 30.9
R.WCTISSPEEKK.C 2: CAM (C), 10: ZEN-CMO (K)	57.2	25.9	- 31.3
K.CLFKVR.E 1:CAM, 4: ZEN-CMO (K)	60.1	29.7	- 30.4
R.KDSNVNWNNLKGK.K 11: ZEN-CMO (K)	56.2	26.6	- 29.6
K.DSNVNWNNLKGK.K 10: ZEN-CMO (K)	61.5	27.9	- 33.6
R.QPVDNYKTCNWAR.V 7: ZEN-CMO (K), 9:CAM (C)	58.4	26.9	- 31.5
R.IQWCAVGKDEK.S 4: CAM (C), 8: ZEN-CMO (K)	58.6	27.6	- 31.0
K.DSAIMLKR.V 7: ZEN-CMO (K)	60.9	28.9	- 32.0
R.KDSNVNWNNLK.G 1: ZEN-CMO (K)	57.6	25.8	- 31.8
K.DLTKCLFK.V 4: ZEN-CMO (K), 5: CAM (C)	64.4	31.5	- 32.9
K.DGKGDVAFVK.H 3: ZEN-CMO (K)	61.2	27.6	- 33.6
K.KGTEFTVNDLQGK.T 1: ZEN-CMO (K)	58.9	26.6	- 32.3

K.SKFMMFESQNK.D 2: ZEN-CMO (K)	62.5	32.0	- 30.5
R.SAGWNIPIGTLLHR.G 1: ZEN-CMO (S)	65.4	35.6	- 29.8
R.EGTTYKEFLGDKFYTVISSLK.T 12: ZEN-CMO (K)	68.0	40.6	- 27.4
K.EFLGDKFYTVISSLK.T 6: ZEN-CMO (K)	71.1	41.6	- 29.5
K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K)	65.9	36.1	- 29.8
R.QCKGDPKTK.T 2: CAM (C), 3: ZEN-CMO (K), 7: ZEN-CMO (K)	64.6	33.7	- 30.9
K.GDPKTKCAR.N 4: ZEN-CMO (K), 6: ZEN-CMO (K), 7: CAM (C)	65.5	34.4	- 31.1

It could be determined that peptides on the UPLC instrument elute with a RT 31.2 min (± 1.4 min) less than on the nLC instrument (compared to gradient B). This occurs due to the higher flow rate on the UPLC instrument, the different gradient and the shorter analysis time.

3.5 Development of a quantification method

After the ZEN-CMO-CON conjugates were thoroughly characterized, the quantification method could be developed.

Conjugate characterization was performed on the HCT plus (Bruker Daltonics, Billerica, MA, USA), the actual quantification method development was then performed on the LC-MS-8030plus (Shimadzu, Manchester, UK) due to its ability for targeted approaches and a higher linear range for quantification compared to the QIT.

As described in chapter 3.3, ZEN-CMO modification is not exclusive for one AA, but widely spread over the protein sequence targeting moreover different types of AAs. This made the development of the quantification method more complicated. If a single AA on the protein is modified, the single peptide in its modified and unmodified state is measured and peak areas can be compared for quantification. In the actual case however many possibilities for modified peptides exist. To get exact results, all modified peptides would have to be measured in the quantification method. It is not assured, that all modification sites were determined in nLC-nESI-QIT measurements. Other Lys residues cannot be ruled out as modification sites, even if they

were not identified. By using QQQ-MS this problem can be handled by using a targeted approach.

Different scan methods were taken into account to find the best suited one for ZEN-CMO quantification. Neutral loss scan and precursor ion scan are suited to address the complexity of a ZEN-CMO modified protein digest, since they are semi targeted approaches that may identify all modified peptides in the sample. For MRM it has to be exactly established what is searched for (precursor ion plus product ion). It is however very specific and sensitive and was therefore also considered as an adequate scan mode.

For the development of a quantification method several parameters were still unknown, e.g. reporter ions and CE. The quantification method was developed on an UPLC—ESI-QQQ-instrument. Therefore sample analysis had to be transferred from the nLC-nESI-QIT to this instrument. This includes adaption of the LC part, CID and identification of reporter ions for targeted analysis. In UPLC higher pressure is possible and the analysis times can be shorter than in nLC. Since the application of CE works differently in a QQQ than in a QIT (fixed CE instead of ramping), reporter ions, which were identified on the QIT could not simply be used for this method. A suitable CE for peptide fragmentation had to be determined. For a successful method transfer systematic investigations of free ZEN-CMO and a ZEN-CMO modified peptide were performed. The spectra were investigated for possible reporter ions for the different scan modes.

3.5.1 Fragmentation study of ZEN-CMO

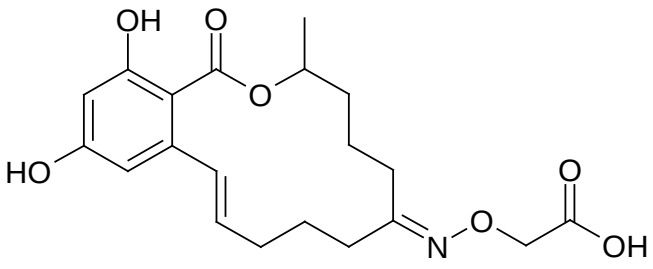


Figure 38 Chemical structure of ZEN-CMO ($C_{20}H_{25}NO_7$). Monoisotopic mass: 391.16 Da

Figure 38 shows the chemical structure of ZEN-CMO. To find suitable fragment ions of ZEN-CMO, which could be used as product ions for the different scan modes, namely precursor ion scan, neutral loss scan and MRM, the fragmentation pattern of the ZEN-CMO modification on peptides had to be studied on the QQQ instrument.

In the course of this study a peptide was ZEN-CMO modified (see chapter 2.2.3). Since the peptide was not purified, free ZEN-CMO was still contained in the peptide solution after conjugate synthesis. This free ZEN-CMO was measured on the QQQ and a product ion scan was performed. 5 µl of the peptide solution were injected for measurement. The amount of free ZEN-CMO, which was contained in the solution is however unknown.

ZEN-CMO has a monoisotopic molecular mass of 391.16 Da. The protonated form of ZEN-CMO therefore has a monoisotopic mass of 392.17 Da. Due to the mass accuracy achievable on a quadrupole analyzer (100 ppm)[48] the integer m/z value of 392 was chosen for product ion scans. A starting CE of -15 V was applied. Figure 39 shows the product ion scan spectrum for ZEN-CMO.

The peak at m/z 392 is corresponding to the still intact protonated ZEN-CMO molecule. The peak at m/z 374 fits to the protonated ZEN-CMO molecule minus 18, which describes the loss of H_2O. The peak at m/z 316 is corresponding to an imine form of the ZEN molecule, which is produced due to cleavage of the bond between the ZEN and the CMO molecule. The peaks at m/z 175, m/z 203 and m/z 316 were described already in Figure 31. It was, thereby verified that these reporter ions can also be used in QQQ experiments to indicate ZEN-CMO modifications (see chapter 3.3). Putative structures for these fragment ions are already given in Figure 34. Possible structures for further ZEN-CMO fragment ions are shown in Figure 40. At this point it was interesting to observe that the fragmentation of ZEN-CMO leads to the formation of an abundant peak at m/z 203.

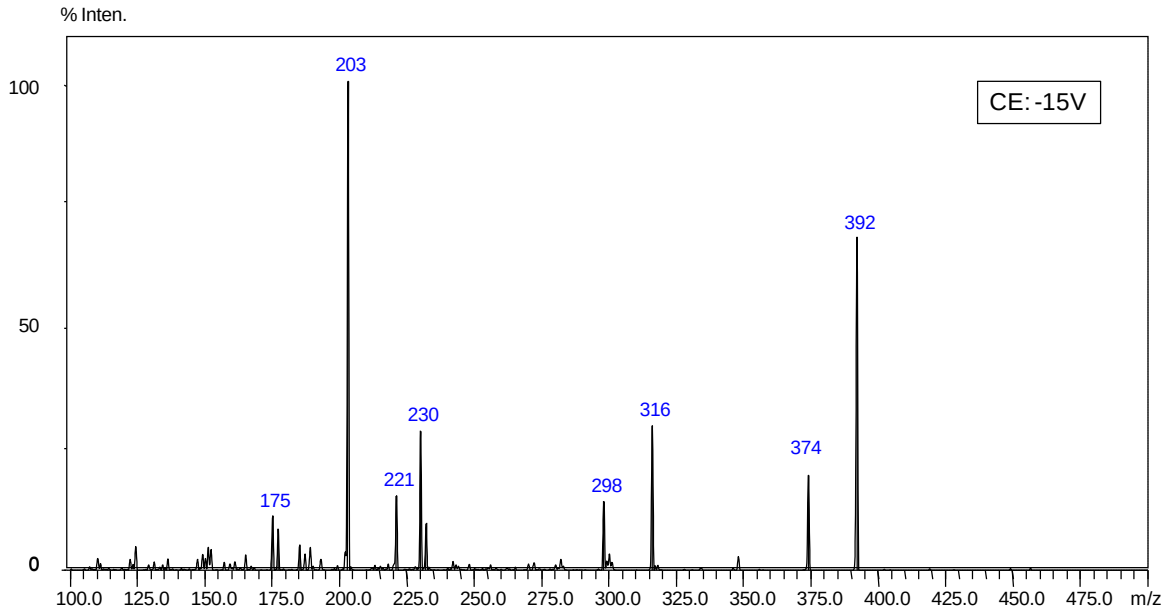


Figure 39 Product ion scan of the protonated form of ZEN-CMO (m/z 392). RT 8.5-9.6 min. CE = -15 V

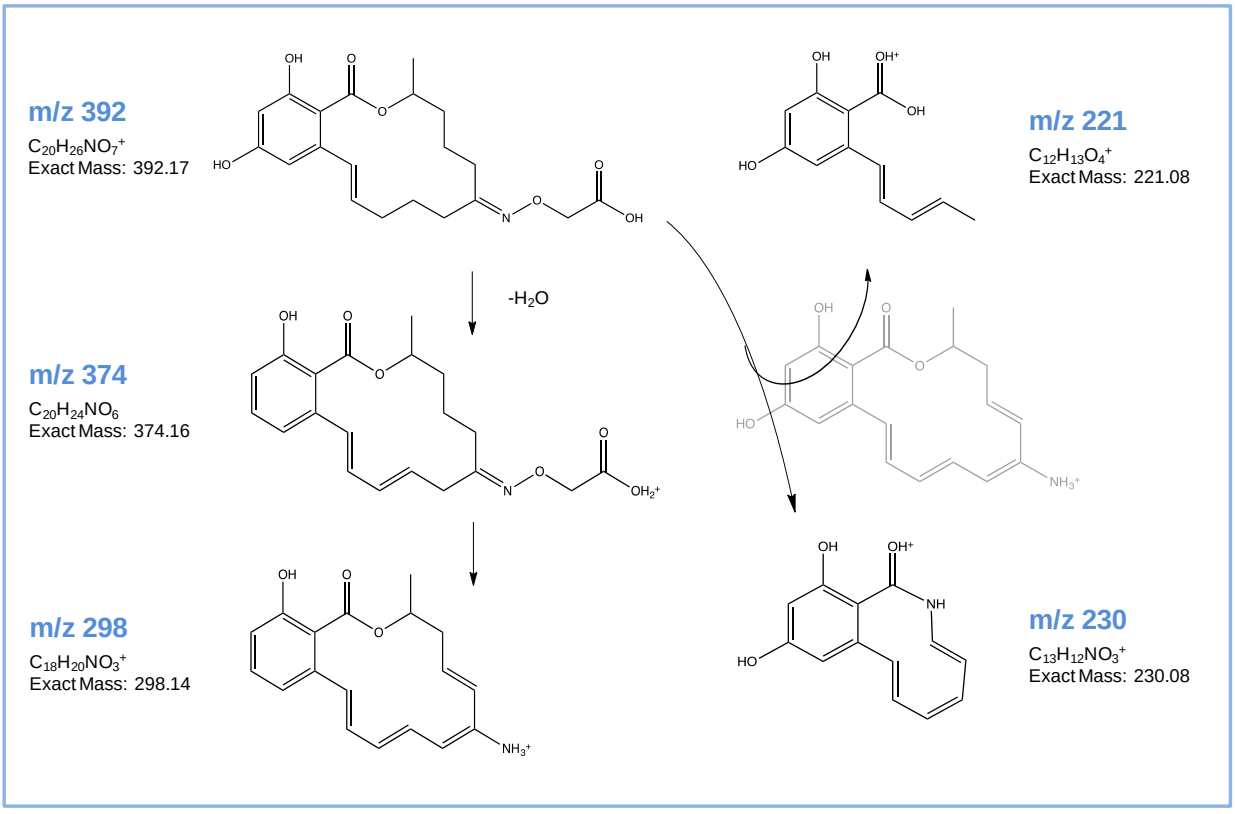


Figure 40 Possible structures for ZEN-CMO fragment ions. For further structures see Figure 34.

3.5.2 Fragmentation of a ZEN-CMO modified peptide

The next important point was to determine a suitable CE, which could be applied for peptide fragmentation. Although it is known that not all peptides need the same fragmentation energy, we wanted to test the specificities of fragmentation at different energy levels for a well-defined peptide containing ZEN-CMO modifications to gain a better understanding of observed mass spectra.

Therefore a peptide with the sequence KRTLRR (monoisotopic mass: 828.54 Da) was ZEN-CMO modified. According to the results of C.Stephan[91], the peptide was expected to mostly carry one modification. Peptide modification is described in chapter 2.2.3. This way the fragmentation behavior of a single peptide could be monitored. 0.05 µg of the modified peptide were measured, which correlates to 60.5 pmol. Since no separation was needed, a short gradient of 20 minutes was applied (see gradient D, chapter 2.5.3).

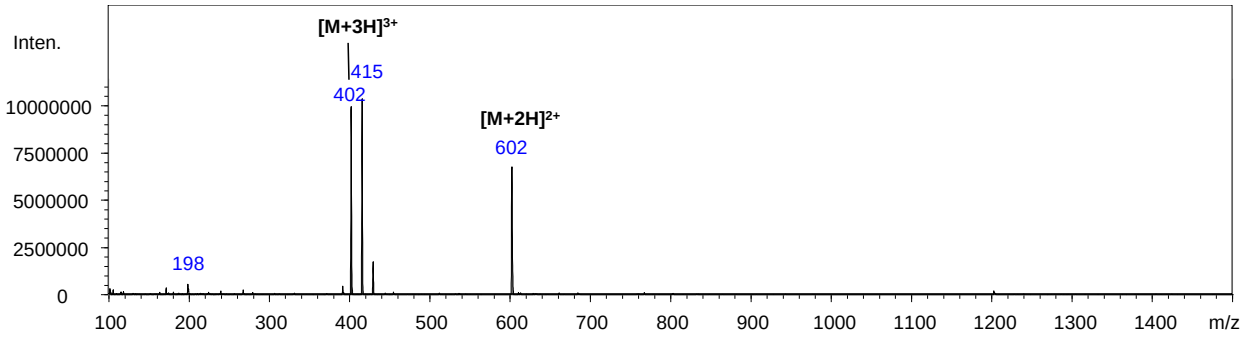


Figure 41 Q1 scan of ZEN-CMO modified peptide KRTLRR. (MS spectrum, RT = 6.1 min., 60.5 pmol). m/z 198 is a background ion

Q1 scan was performed to determine the charge state of the modified peptide. The modified peptide carrying one ZEN-CMO modification could be detected at RT = 6.1 min (see Figure 41). The peak at m/z 402 is corresponding to the triply charged ZEN-CMO modified peptide ($[M+3H]^{3+}$ at m/z 401.6), while the peak at m/z 602 correlates to the doubly charged peptide ($[M+2H]^{2+}$ at m/z 601.8). Also a very intense peak at m/z 415 is visible in the spectrum. The m/z value corresponds to the doubly charged unmodified peptide ($[M+2H]^{2+}$ at m/z 415.3). This is however unlikely since the modified peptide is more hydrophobic than the unmodified, leading to stronger retention on the column and preventing co-elution of the peptides (as described for

CON peptides in chapter 3.4). Therefore they will not elute at the same time. This ion is maybe resulting from in-source fragmentation.

This peptide represents a suitable model peptide as most of the identified peptides in the ZEN-CMO conjugate digest actually carry only one ZEN-CMO modification. Further analysis focused on CEs and the fact that the modified peptide was detected in its doubly and triply charged state. These abundant m/z values were chosen for fragmentation experiments.

Fragmentation behavior of the triply charged ZEN-CMO modified peptide

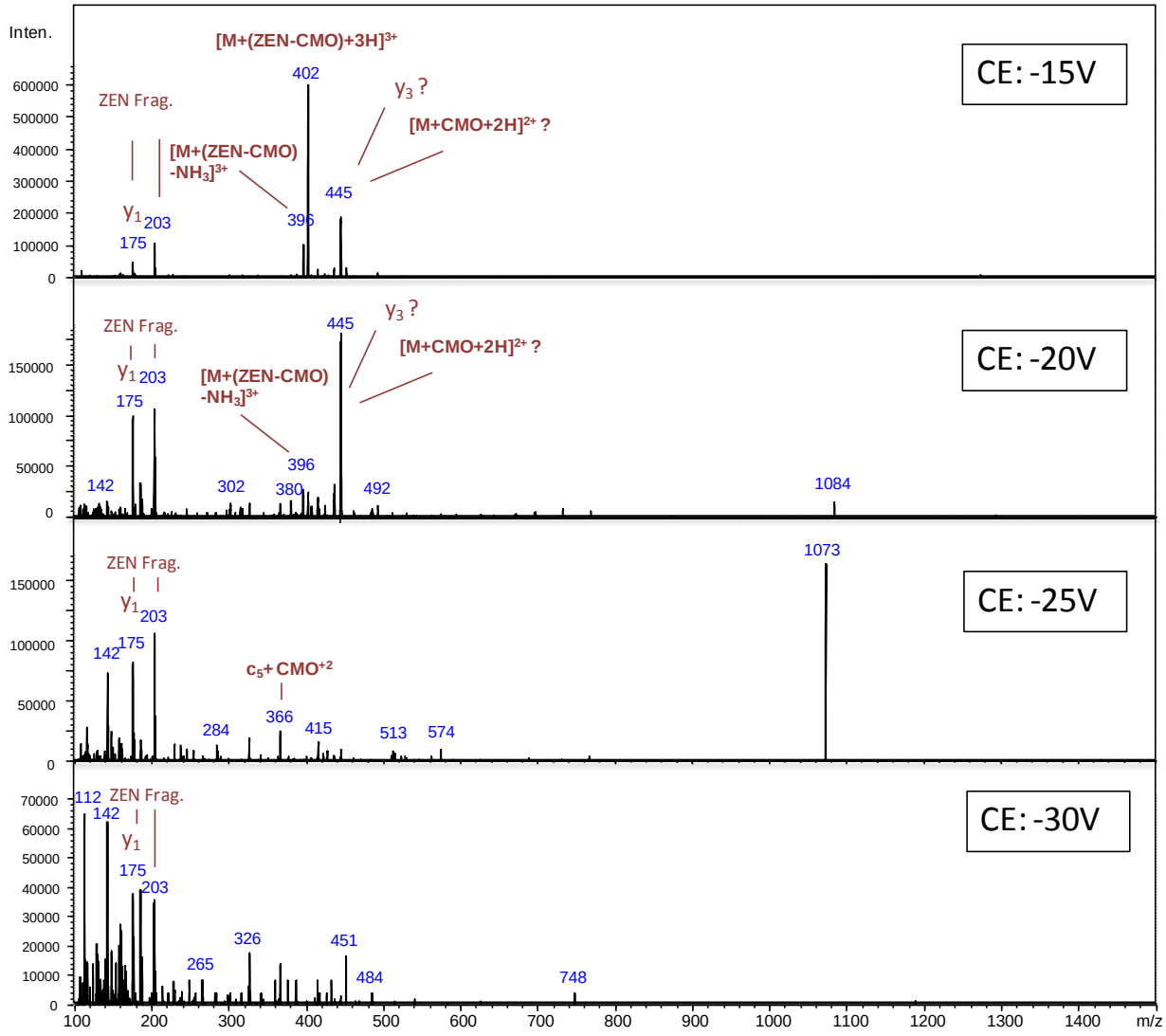


Figure 42 Product ion scan of the triply charged ZEN-CMO modified peptide KRTLRR ([M+3H]$^{3+}$ at m/z 402). CE was varied between -15 V and -30 V. Fragment ions are tentatively assigned.

At first the triply charged peptide (m/z 402) was chosen for fragmentation. Product ion scan was performed with CEs of -15 V, -20 V, -25 V and -30 V (see Figure 42). At -15 V the precursor was barely fragmented and is still the most abundant peak in the spectrum. However the signals at m/z 175 and m/z 203, which belong to singly charged fragments of the ZEN-CMO modification, are already visible. Also a peak at m/z 445 appears in the spectrum, which is tentatively assigned to the y_3^+ ion of the peptide (m/z value of 444.3). Another possible explanation could be that this signal is corresponding to the doubly charged peptide that already lost the ZEN molecule. If only the CMO part is attached to the peptide, its mass increases by 58 Da. The doubly charged modified peptide with the CMO linker still attached would therefore result in an m/z value of 444.3 as well. However, both values are not perfectly matching the measured value. Nevertheless, the rather low mass accuracy of the QQQ allows for the explanations that the peak at m/z 445 is corresponding to either ion. At a CE of -20 V the precursor ion was nearly fully fragmented.

Application of the different fragmentation energies showed that the ZEN-CMO fragments of m/z 175 and m/z 203 were produced in all experiments, though the intensity of the fragment ion m/z 203 started to decrease at CE -30 V. Further fragmentation of this ion might be an explanation.

Fragmentation of the doubly charged ZEN-CMO modified peptide

In the next step the doubly charged peptide was chosen as precursor (m/z 602) and product ion scan was performed at CEs of -20 V, -25 V, -30 V and -35 V. 60.5 pmol were injected for measurement. The spectra are shown in Figure 43.

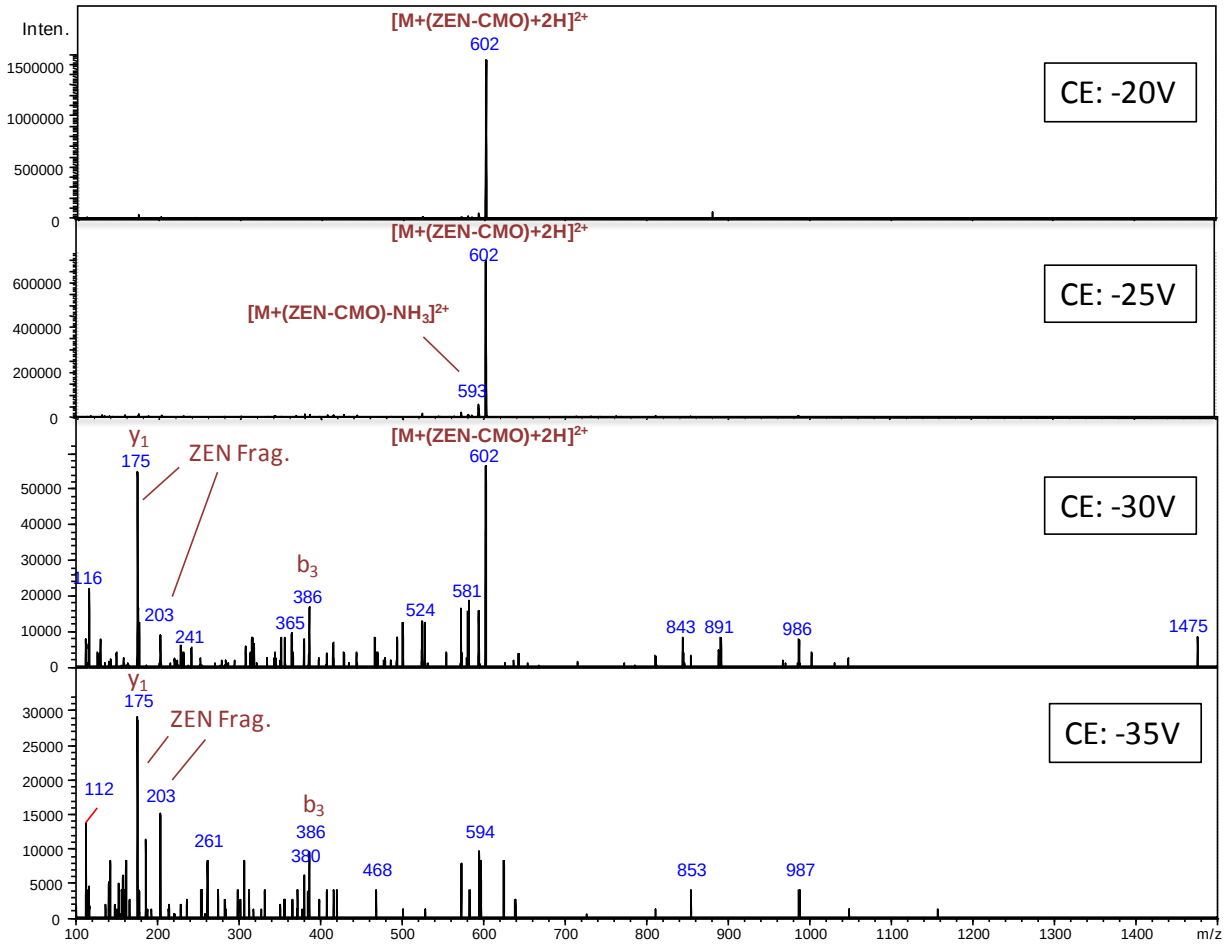


Figure 43 Product ion scan of the doubly charged ZEN-CMO modified peptide KRTLRR (m/z 602). CEs from -20 V up to -35 V were applied.

In the case of the doubly charged peptide the precursor ion did not fully fragment at CEs between -20 V and -25 V. Only at -30 V fragment ions became clearly visible in the spectrum. The product ions 175 and 203 of the ZEN-CMO modification were formed at all CEs.

Fragmentation studies of the ZEN-CMO modified peptide lead to the conclusion, that peptide charge influences fragmentation behavior. While the triply charged peptide could already be partly fragmented at a CE of -15 V the doubly charged peptide did not give reasonable spectra up to a CE of -30 V. If a peptide is carrying a higher number of charges it becomes more "instable" and a lower CE already induces fragmentation.[97] The ZEN-CMO characteristic fragments m/z 175 and m/z 203 are frequently produced as soon as the modified peptide starts fragmenting.

The goal of this study was to find a suitable CE for the fragmentation of ZEN-CMO modified peptides generated in a ZEN-CMO modified protein digest. Since peptides very often occur as

doubly as well as triply charged ions, a CE is needed that would fit both types, so that one CE can be applied during the analysis. At this point it has to be said, that for best results, CEs should be adapted for all peptides of interest, since it is possible to change the CE for a certain RT.

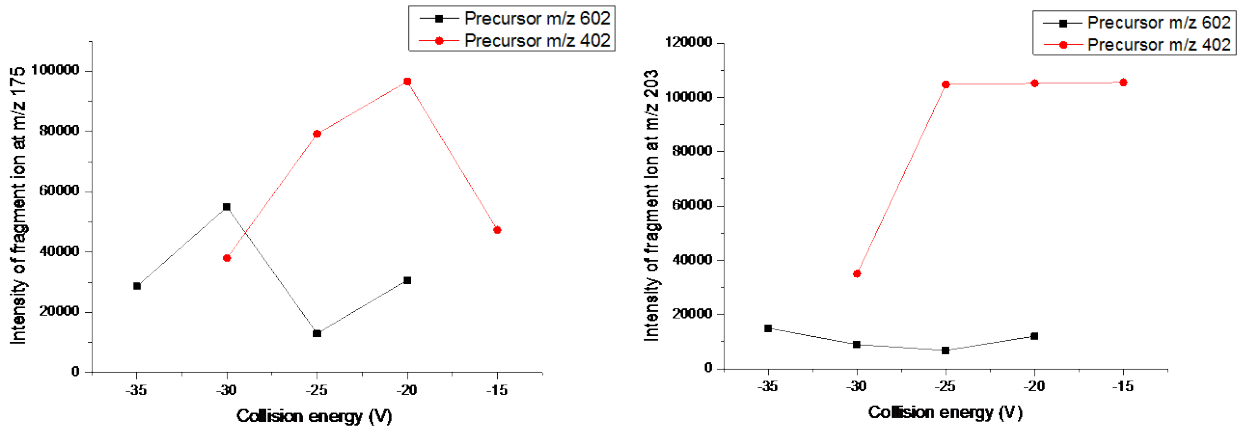


Figure 44 Intensity of fragment ions m/z 175 and m/z 203 at different CEs for the doubly (m/z 602) and triply (m/z 402) charged precursor ion

The ZEN-CMO characteristic fragments occur at all CEs. It was observed that the intensity of these ions however decreased with higher CEs. This can be explained by unspecific fragmentation due to secondary collisions. Since the CE was not adapted for each peptide a higher CE of -30 V was chosen to make sure that the precursor would fragment. It was to be expected that this CE would be high enough for the doubly charged peptides to result in reasonable fragmentation, while the triply charged peptide would not be to much fragmented. It appears however that the ZEN-CMO modification fragments at lower CEs than the peptide backbone itself.

3.5.3 Reporter ions

Since it was of interest to find a QQQ scan method that would be best suited for quantification of the ZEN-CMO modification, product ions formed by the modification were needed for product ion scan and MRM.

As seen in the spectra in Figure 42 and Figure 43 the fragment ions m/z 175 and m/z 203 were occurring on a regular basis and can be observed in all spectra independent of the CE. Therefore these ions seemed to be good candidates as product ions.

For the application of the quantification method it was of desire to find a reporter ion, which would be specific for the modification, to avoid false positive results. To evaluate the suitability of the product ions, certain pitfalls have to be taken into account.

False positive results can occur, if an unmodified peptide in the sample forms a fragment ion having the same m/z value as the indicative ZEN-CMO product ion. For this issue one can say that the possibility of a false positive result is reduced if MRM is applied. In this case false positive results can only occur, if the modified and unmodified peptide have the same m/z value and moreover give the same fragment ions, besides the fact that these two peptides would not elute at the same retention time.

Nevertheless for completeness, possible AA combinations of tryptic CON peptides were considered, giving fragment ions of m/z 175 and m/z 203.

m/z 175 had to be rejected immediately as a suitable reporter ion, since this m/z value equals the y_1-ion of a tryptic peptide containing Arg on the C-terminus. Considering the fact that exactly those peptides are easier ionized and therefore more often detected than Lys containing ones makes this reporter ion an even worse choice. Further the combination of Asp and Ser on the N-terminal end of a peptide can result in an *a*-ion of m/z 175. This combination occurs 3 times in the CON sequence after a potential tryptic cleavage site.

Some sequences in CON also give rise to a fragment ion fitting m/z 203. Possible a-, b-, c-, x-, y- and z- fragment ions that can be produced by CID fragmentation of a tryptic CON peptide were investigated (a maximum of 2 missed cleavages was considered).

Table 28 Tryptic CON peptides (max. 2 missed cleavages) giving rise to a fragment ion m/z value 203

AA combi.	ion type	Peptide sequence
Ala + Asn	c_2-ion [AA residue+18]	**[185 – 199] R.*NA*PYSGYSGAFHCLK.D** **[185 – 202] R.*NA*PYSGYSGAFHCLKDGK.G** **[185 – 209] R.*NA*PYSGYSGAFHCLKDGKGDVAFVK.H** **[576 – 582] R.*AN*VMDYR.E** **[576 – 600] R.*AN*VMDYRECNLAEVPTHAVVVRPEK.A** **[576 – 603] R.*AN*VMDYRECNLAEVPTHAVVVRPEKANK.I** **[601 – 603] K.*AN*K.I** **[601 – 605] K.*AN*KIR.D** **[601 – 610] K.*AN*KIRDLLER.Q**
Asp+Asp	a_2-ion [AA residue -27]	**[256 – 259] R.*DD*NK.V** **[256 – 269] R.*DD*NKVEDIWSFLSK.A** **[256 – 279] R.*DD*NKVEDIWSFLSKAQSDFGVDTK.S**
Asp + Ser	b_2-ion [AA residue +1]	**[302 – 308] K.*DS*AIMLK.R** **[302 – 309] K.*DS*AIMLKR.V** **[302 – 332] K.*DS*AIMLKRVPSLMDSQLYLGFEYYSAIQSMR.K** **[440 – 449] K.*DS*NVNWNNLK.G** **[440 – 451] K.*DS*NVNWNNLKGK.K** **[440 – 452] K.*DS*NVNWNNLKGKK.S** **[280 – 290] K.*SD*FHLFGPPGK.K** **[280 – 291] K.*SD*FHLFGPPGKK.D** **[280 – 296] K.*SD*FHLFGPPGKKDPVLK.D**
Gly + Lys	c_2-ion [AA residue +18]	**[100 – 112] K.*KG*TEFTVNDLQGK.T** **[100 – 121] K.*KG*TEFTVNDLQGKTSCHTGLGR.S**

As shown in Table 28, formation of product ions of m/z 203 is not completely unlikely either. However the possibility that a m/z 203 fragment ion is detected is still far lower than detecting m/z 175. Still false positive results might occur during the product ion scan. Therefore it has to be confirmed, if ions, detected in a precursor ion scan, actually correspond to a modified peptide or not. As explained before, this is only a problem for MRM, if the m/z value of a modified peptide is equal to an unmodified peptide, which produces a fragment ion of m/z 203.

Table 29 Possibility of a peptide in CON (max. 2 missed cleavages) giving a false positive result

m/z value	Number of unmodified peptides in CON giving rise to the m/z value
175	96
203	23
175+203	13

14 tryptic peptides resulting from a digest of CON can lead to an ion of the same m/z value as a ZEN-CMO-modified peptide. 7 of them produce a fragment ion of m/z 175 and 3 peptides even produce fragment ions of both m/z 175 as well as m/z 203. These peptides are shown in Table 30.

Table 30 ZEN-CMO modified and unmodified peptides forming an ion with the same m/z value. Unmodified peptides producing reporter ions with m/z 175 and m/z 203 displayed in italic

m/z	Sequence	RI m/z 175	RI m/z 203
311.8^{3+}	**R.QEKR.F** 3: ZEN-CMO (K)	x	x
311.8^{3+}	***K.DSAIMLKR.V***		
353.2^{2+}	**K.ANK.I** 3: ZEN-CMO (K)		
353.2^{2+}	***K.GKK.S*** 2: ZEN-CMO (K)		
353.2^{3+}	**K.FYTVISSLK.T**		
389.2^{2+}	**R.QEK.R** 3: ZEN-CMO (K)	x	x
389.2^{2+}	***K.DSAIMLK.R***		
404.2^{2+}	**R.FGVNGSEK.S** 8: ZEN-CMO (K)	x	
404.2^{3+}	***K.TCNWAR.V*** 2: CAM(C)		
404.2^{1+}	**R.QEK.R**		
412.9^{3+}	**R.QPVDNYK.T** 7: ZEN-CMO (K)		
412.9^{3+}	**R.WCTISSPEEK.K** 2: CAM (C)		
432.7^{2+}	**R.DDNK.V** 4: ZEN-CMO (K)		
432.7^{2+}	**K.LCRQCK.G** 2: CAM (C), 5: CAM (C)		
463.6^{3+}	**K.KSCHTAVGR.T** 1: ZEN-CMO (K), 3: CAM (C)	x	x
463.6^{3+}	**K.DSNVNWNNLKGK.K**		
480.9^{3+}	**K.AQSDFGVDTK.S** 10: ZEN-CMO (K)	x	

480.9^{3+}	***R.KDQLTPSPRENR.I***		
534.3^{2+}	**K.ANKIRDLLER.Q** 3: ZEN-CMO (K)		
534.3^{3+}	**K.AQSDFGVDTK.S**		
551.6^{3+}	**K.DLTKCLFKVR.E** 5: CAM (C), 4: ZEN-CMO (K)	x	
551.6^{3+}	***K.TDERPASYFAVAVAR.K***		
561.3^{3+}	**K.GTEFTVNDLQGK.T** 12: ZEN-CMO (K)	x	
561.3^{3+}	***K.TCNWARVAAHAVVAR.D*** 2: CAM (C)		
618.8^{2+}	**R.QPVDNYK.T** 7: ZEN-CMO (K)		
618.8^{2+}	**R.WCTISSPEEK.K** 2: CAM (C)		
699.4^{2+}	**K.DLTKCLFK.V** 5: CAM (C), 4: ZEN-CMO (K)		
699.4^{1+}	**K.KDPVLK.D**		
777.4^{1+}	**R.QEK.R** 3: ZEN-CMO (K)	x	x
777.4^{1+}	***K.DSAIMLK.R***		
826.9^{2+}	**K.DLTKCLFKVR.E** 5: CAM (C), 4: ZEN-CMO (K)	x	
826.9^{2+}	***K.TDERPASYFAVAVAR.K***		
841.4^{2+}	**K.GTEFTVNDLQGK.T** 12: ZEN-CMO (K)	x	
841.4^{2+}	***K.TCNWARVAAHAVVAR.D*** 2: CAM (C)		
864.4^{1+}	**R.DDNK.V** 4: ZEN-CMO (K)		
864.4^{1+}	**K.LCRQCK.G** 2: CAM (C), 5: CAM (C)		
933.5^{1+}	**R.QEKR.F** 3: ZEN-CMO (K)	x	x
933.5^{1+}	***K.DSAIMLKR.V***		
961.5^{2+}	**R.IQWCAVGKDEKSK.C** 4: CAM (C), 8: ZEN-CMO (K)		
961.5^{1+}	**R.IQWCAVGK.D** 4: CAM (C)		
1236.6^{1+}	**R.QPVDNYK.T** 7: ZEN-CMO (K)		
1236.6^{1+}	**R.WCTISSPEEK.K** 2: CAM (C)		
1388.7^{1+}	**K.KSCHTAVGR.T** 1: ZEN-CMO (K), 3: CAM (C)	x	x
1388.7^{1+}	***K.DSNVNWNNLKGK.K***		

3.5.4 Comparison of scan modes

As explained in chapter 1.6.3, several scan modes can be used on a QQQ instrument. To find the best suited scan method for the quantification of ZEN-CMO modifications, neutral loss scan, precursor ion scan and MRM were investigated as possible scan modes.

3.5.4.1 Neutral loss scan

The consideration for a successful implementation of the neutral loss scan was that the easy fragmentation of the nitrogen-oxygen bond between ZEN and CMO is observed rather often. Breaking of this bond would lead to a loss of the ZEN molecule (316.15 Da), while the CMO part of the modification would stay attached to the charged peptide, resulting in a neutral loss of 315.15 Da. Thereby a constant mass difference of m/z 157.6 and m/z 105.0 would be the result for a doubly or a triply charged peptide respectively.

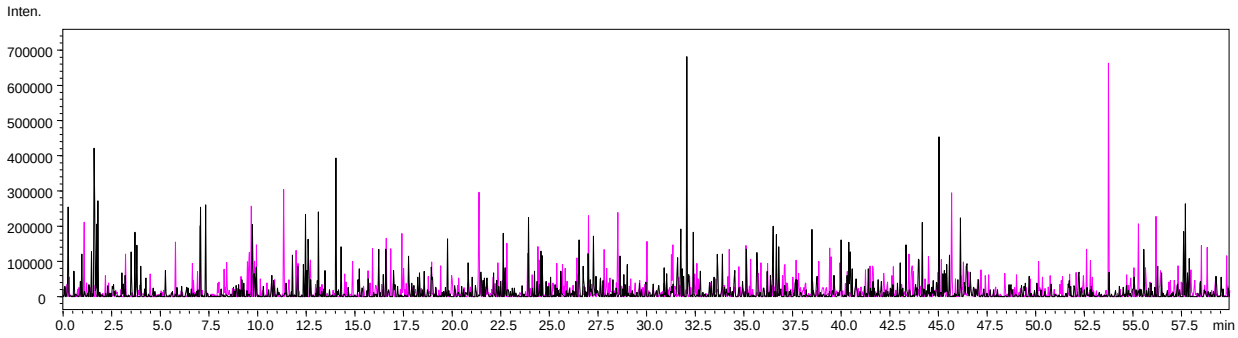


Figure 45 Total ion current (TIC) chromatogram of sample B3 (78 pmol) resulting from a constant neutral loss scan at a CE of -30 V. TIC 1 (black) shows a constant mass difference of m/z 105.0. TIC 2 (pink) shows a constant mass difference of m/z 157.6

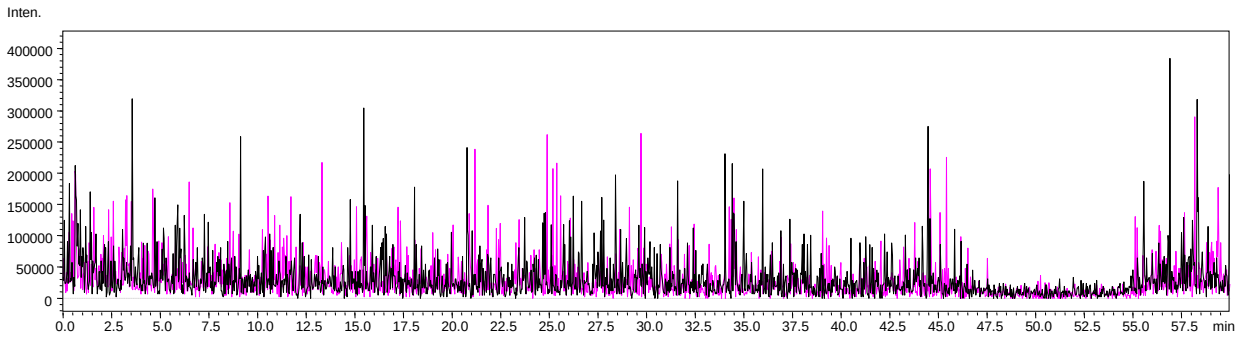


Figure 46 Total ion current (TIC) chromatogram of sample B3 (78 pmol) resulting from a constant neutral loss scan at a CE of -25 V. TIC 1 (black) shows a constant mass difference of m/z 105.0. TIC 2 (pink) shows a constant mass difference of m/z 157.6

In Figure 45 and Figure 46 the results of two different neutral loss scans are displayed. 78 pmol of sample B3 were measured after digestion with trypsin/LysC and desalting with C_{18} spin columns. CE was chosen to be -25 V and -30 V, because these CEs could be used to fragment the test peptide. Constant mass differences of m/z 157.6 and m/z 105.0 were measured. The chromatogram only shows random noise but no clear peak for any modified peptide was detected. Possible explanations for this are that either the desired breaking of the nitrogen-oxygen bond did not occur due to inapt CEs or that the peptide was fragmented beyond the loss of the ZEN molecule and therefore constant mass differences could not be measured. No differences could be observed between CEs of -30 V and -25 V.

Since the various peptides and ions fragment differently at different energies, it is very difficult to find the fragmentation energy leading to a neutral loss without a vast number of experiments narrowing down the optimum CE for each and every modified peptide.

After investigating the MS/MS spectra of the modified peptide KRTLRR, it was concluded that the neutral losses cannot be observed in these spectra either. It appears however that the peptide changes the charge state after the ZEN molecule is lost. If the triply charged peptide is fragmented at low CEs (-15 V - -20 V), a peak corresponding to the doubly charged peptide minus the ZEN molecule can be identified in the spectrum. See chapter 3.5.2.

Therefore neutral loss scan was excluded as a suitable scan method and was not further investigated.

3.5.4.2 Precursor Ion Scan

Since it was of desire to quantify all modified AA residues in a ZEN-CMO-CON digest, precursor ion scan posed as a good scan method for the quantification method.

It was expected that all ZEN-CMO modified peptides would produce a fragment ion of m/z 203 and would thus be detected in the chromatogram. The downside of product ion scan has already been described in chapter 3.5.3. The m/z value 203 is not exclusively formed by the ZEN-CMO modification, but can also be formed by unmodified peptides, which could falsify the results. Therefore each peak should be investigated and confirmed as corresponding to a modified peptide.

ZEN-CMO-CON conjugates were digested with trypsin/LysC, desalted, dried and dissolved with 5% ACN in water / 0.05% FA to a concentration of 7.8 pmol/µl. 10 µl were injected for

measurement of sample B3 (= 78 pmol). Product ion scan was performed by using the ZEN-specific fragment ion at m/z 203 as characteristic product ion.

Figure 47 shows a total ion current (TIC) chromatogram of the precursor ion scan of sample B3. Again noise is dominating the chromatogram and analyte signals are not clearly visible. Registered mass spectra usually show all ions scanned in Q1 when triggered by a fragment ion of m/z 203 detected in Q3, so it cannot be determined with absolute certainty, which precursor ion produced the fragment ion. Thus false positive results are possible. A ZEN-CMO-CON digest delivers a high number of various peptides. Therefore identifying a peptide by its m/z value alone is not very significant.[98]

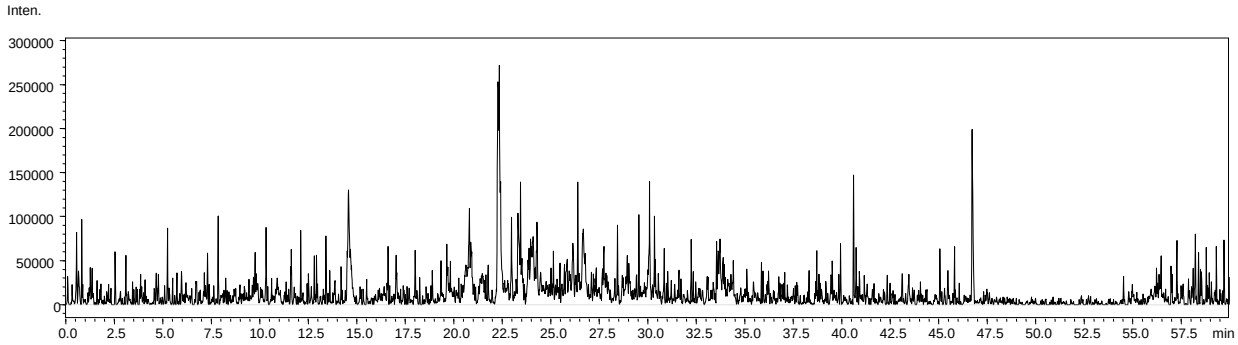


Figure 47 Precursor ion scan TIC of sample B3 (78 pmol). Product ion: m/z 203. CE: -30 V

As an example the MS spectrum of the peak at RT 22.1-22.6 min from Figure 47 is shown in Figure 48. The spectrum shows a peak of m/z 594. This m/z value matches the unmodified triply charged peptide K.TDERPASYFAVAVARK.D ($[M+3H]^{3+}$ at m/z 594.3) as well as the doubly charged unmodified peptide K.DPVLKDLLFK.D ($[M+2H]^{2+}$ at m/z 594.4). However these peptides should not produce a fragment ion of m/z 203. The m/z value also matches the 2 times ZEN-CMO modified, triply charged peptide K.GDPKTKCAR.N ($[M+3H]^{3+}$ at m/z 593.6.

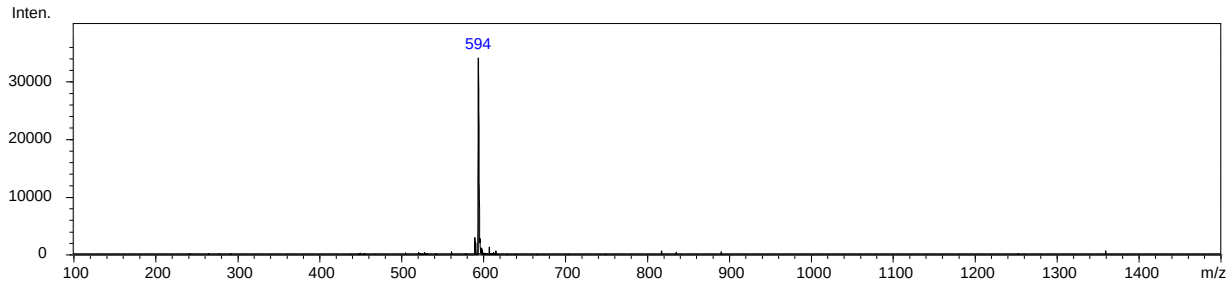


Figure 48 MS Spectrum of peak at 22.1 – 22.6 min in sample measurement B3 (Chromatogram see Figure 47)

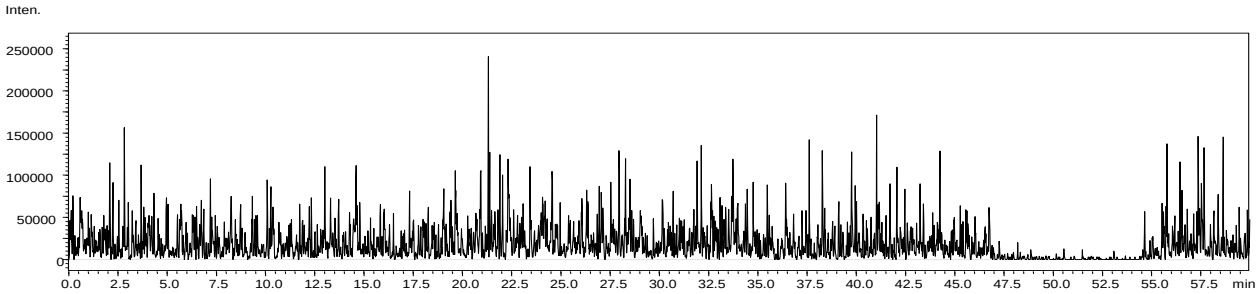


Figure 49 Precursor ion scan TIC of sample A4 (156 pmol). Product ion: m/z 203. CE: -30 V

In Figure 49 a precursor ion scan TIC chromatogram of sample A4 is displayed. In this case again no signals could be detected although a rather high amount of analyte was investigated (156 pmol corresponding to 12 µg unmodified protein). Even though double the amount of sample B3 was measured, the amount is still too low to detect modified peptides in sample A4.

It has to be mentioned that B3 has a higher modification rate, which leads to a higher number of modified peptides. For modification quantification modified peptides need to be detected in samples with a lower modification rate as well. Thus it seems that higher sample amounts are needed to detect ZEN-CMO modifications on samples with a lower rate of modification.

However since sample amounts were limited, it was decided to investigate MRM next, since precursor ion scan turned out to be not well suited due to its low specificity.

3.5.4.3 Multiple reaction monitoring

MRM is a highly sensitive and specific scan method. In MRM scans the precursor as well as the product ions are fixed. Therefore noise is reduced to a minimum and thus the limit of detection is usually lower than in other scan modes. Thus very low amounts of sample can be used for measurements. Also the number of false positive hits is significantly reduced. Thus MRM seemed like a good candidate for the quantification method of choice.

A disadvantage of MRM is however that the approach is targeted and that therefore only predefined substances are detected. In this study ions of the modified peptides, which were identified in the nLC-nESI-QIT measurements, were chosen as precursor ions. Although there might be the possibility that more peptides are modified, the knowledge from the nLC-ESI-QIT experiments was used as basis for method development. As described in chapter 3.3, the detected ZEN-CMO modifications are evenly distributed over the whole protein surface. Thereby

it was hoped that these modified AA residues should give sufficient information on a protein´s modification rate, even if not all ZEN-CMO modified peptides are detected in the measurement. The m/z value 203 was chosen as product ion, due to its high abundance and specificity.

ZEN-CMO-CON conjugates were digested with trypsin/LysC and desalted using C_{18} spin columns. 78 pmol of protein were used for measurement. See chapter 2.5.4 for further information.

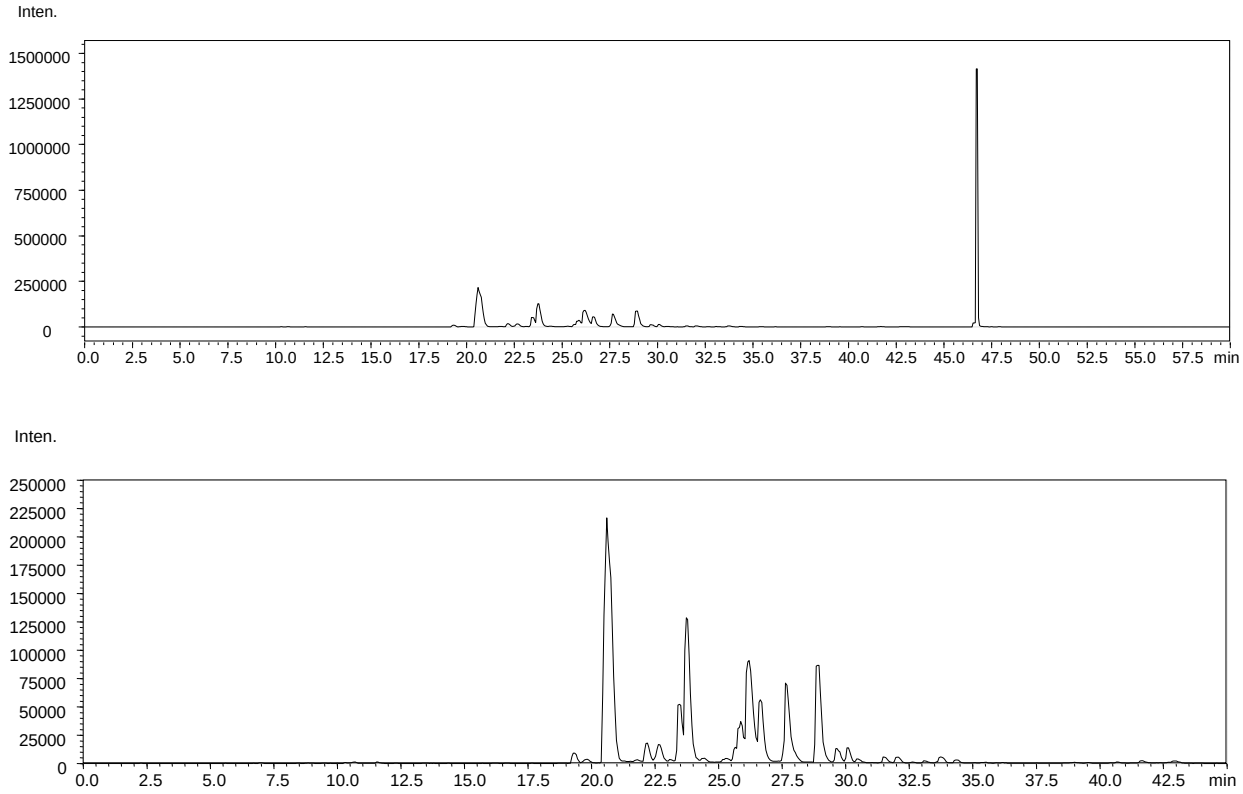


Figure 50 MRM measurement. TIC chromatogram of sample B4 (78 pmol). Product ion: m/z 203, CE: -30 V. Top = full measurement, Bottom = zoomed area between 0 – 45 min

In Figure 50 two chromatograms of sample B4, measured by MRM are displayed. The chromatograms show the same measurement. In the top chromatogram a peak at m/z 598.3 appears at RT 46.7 min. Since this peak occurs after the ACN gradient exceeded 50% it is assumed to be a false positive match. Since the peptides were desalted by binding to C_{18} material and eluted with 50% ACN + 0.05%FA, all peptides have to elute at a maximum of 50% ACN from the LC column. An ion with m/z 598.3 was also detected at RT 29.7 min, which is assumed to be corresponding to the actual peptide K.CLFKVR.E 1: CAM (C), 4: ZEN-CMO (K). In the bottom chromatogram a zoom of the top chromatogram (0 – 45 min) is displayed.

For the first time, peaks are clearly visible and no noise is observable. All 33 MRM transitions could be monitored, therefore the same peptides as in QIT measurements were detected. (See Table 15 for MRM transitions).

Even though MRM is very specific, even in this case false positive results may occur (See Table 29).

To increase sensitivity the number of data points taken per MRM event was increased by defining time frames for each MRM event. By this the Q had more time for the detection of the selected MRM transitions because the number of monitored ions was significantly decreased. This also reduces the possibility of false positive results, since the chance for modified and unmodified peptides having the same m/z value to elute at the same time are considered as rather unlikely. To check if the peaks are really corresponding to the modified peptides, UPLC RTs were compared to RTs of the modified peptides on the nLC instrument (See Table 27).

It was concluded that MRM is the best-suited scan method for ZEN-CMO quantification. It seems to be the only scan method that can provide the sensitivity needed for the detection of the modified peptides and the only method that provides appropriate peaks for integration. To confirm this assumption, dilutions of the sample should be measured to determine the limit of detection. Further it is more specific than the other methods and false positive identifications are omitted.

Therefore MRM was used for the quantification of ZEN-CMO modifications. For detailed settings of the method see chapter 2.5.4.

3.5.5 Quantification measurements

After MRM was determined as the best suited method for quantification, all ZEN-CMO-CON conjugates, which were provided by Romer Labs, were digested using trypsin/LysC mix and desalted with C_{18} spin columns. For further information on the samples see chapter 2.2.1.

78 pmol of each sample were used for measurement. CID was performed at -30 V. Additionally unmodified CON was measured as blank. In Figure 51 a measurement of an unmodified CON is displayed. No significant peak, induced by the pre-defined MRM transitions for the modified peptides, was detected.

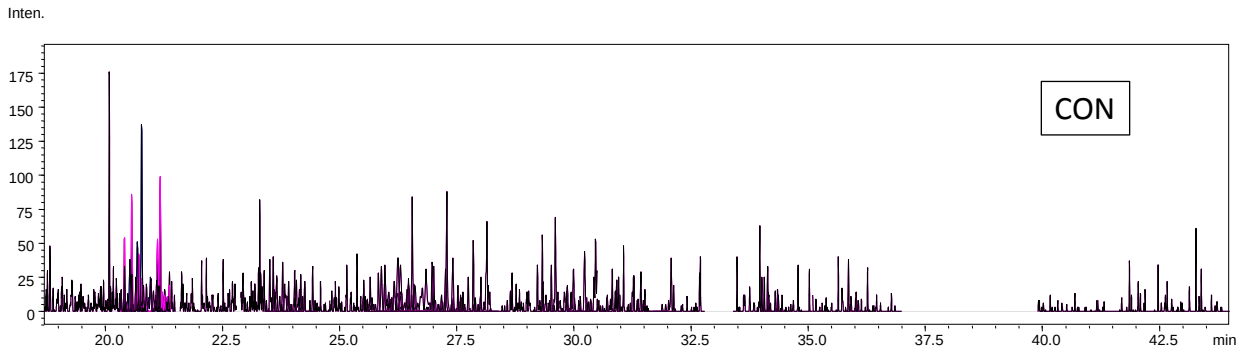


Figure 51 TIC chromatogram of MRM measurement of unmodified CON for comparison as blank (different colors occur if different measurements are set in the same time frame)

To test the methods reproducibility, sample A3 was independently digested twice. Peak areas were summarized and compared. It was found that the results differ by 10 % as the ratio of the sum over all peak areas was 1 : 1.1. This is at the moment interpreted as a very good result over the whole method, covering sample digestion, desalting, peptide clean up and LC-MS/MS measurement. Since not all molecules of a protein in one sample carry the same amount of ZEN-CMO modifications, this might influence the result.

Figure 52 shows MRM measurements for samples A. Sample A1 shows only very weak peaks, which cannot be distinguished from noise. So it cannot be said with absolute certainty that these peaks are not produced by noise, since even in the unmodified sample small peak areas can be detected. It is therefore concluded that sample A1 is not modified or that the modification rate is very small. Most peptides in this sample supposedly do not exceed the limit of detection for the modified analogue. Since all samples termed with an “A” have the same activation time, differing only in the molar ratio for ZEN-CMO coupling, it can be concluded that at an activation time of 1 hour, cr has to be above 1:10 for decent modification detection. The average ZEN-CMO modification for this sample in MALDI measurements was 0 ZEN-CMO per CON molecule according to unpublished data by Sophie Fröhlich (2013). It was not possible to detect all 33 MRM transitions (see Table 15) in all sample measurements. In the sample measurement for A1 11 peaks were measured, in A2 14 peaks, in A3 18 peaks and in sample A4 24 peaks. The peaks in sample A1 also appear in the measurements of the other samples. The higher modified the sample the more additional peptides were detected in the measurement. The peak areas detected in sample A1 are however very small, and it is likely that they are produced by noise. It would be false to conclude that the detected modified peptides are the preferred sites of modification. Peak areas of different substances cannot be compared for quantification. Different peptides have different ionization efficiencies; thus some are more easily detected and show

higher intensity and peak areas than others, even if an equimolar amount of both substances is included in the sample. Therefore some peptides were only detected with increased modification rate.

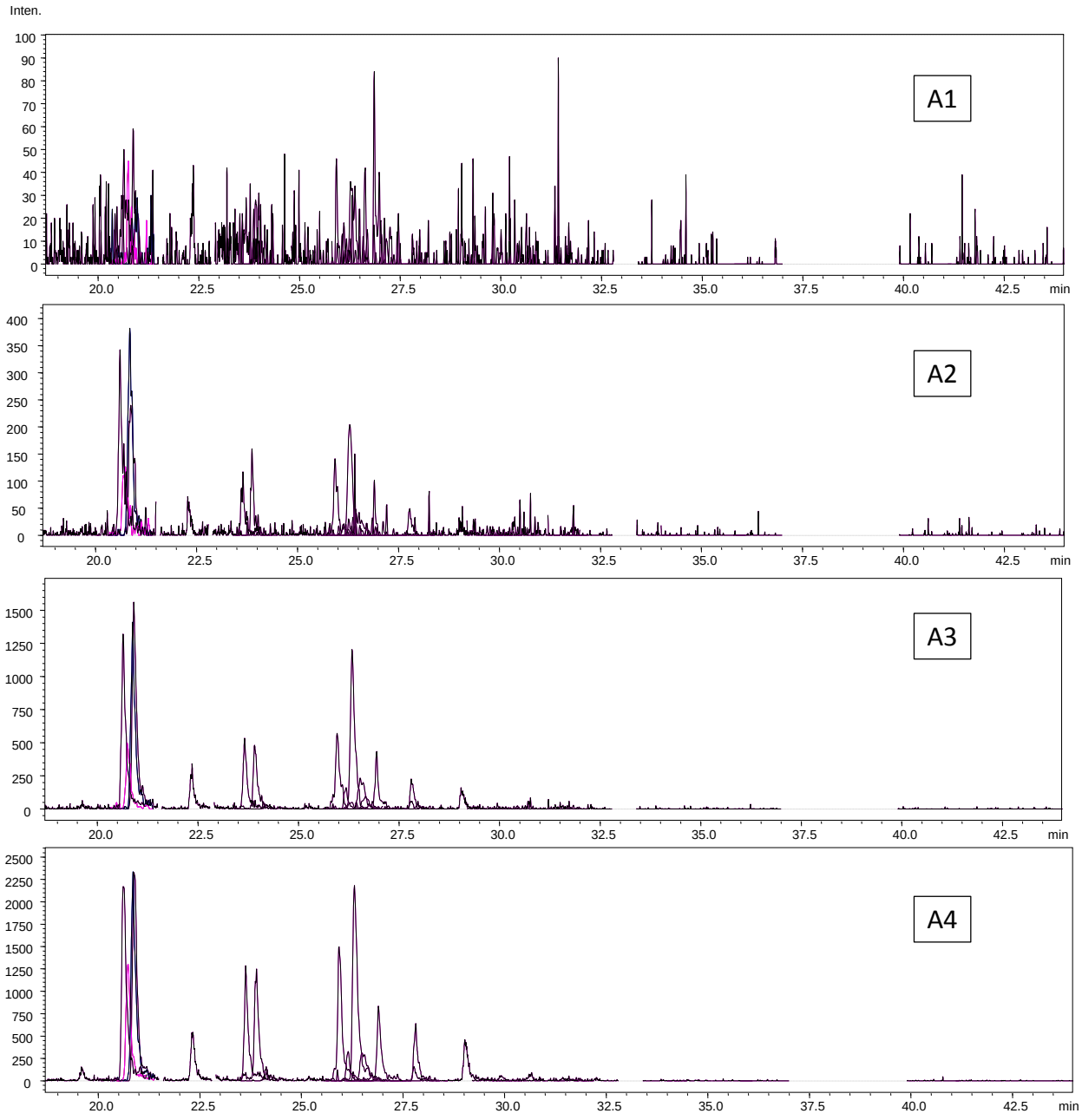


Figure 52 TIC chromatograms of MRM measurements of samples A1, A2, A3 and A4

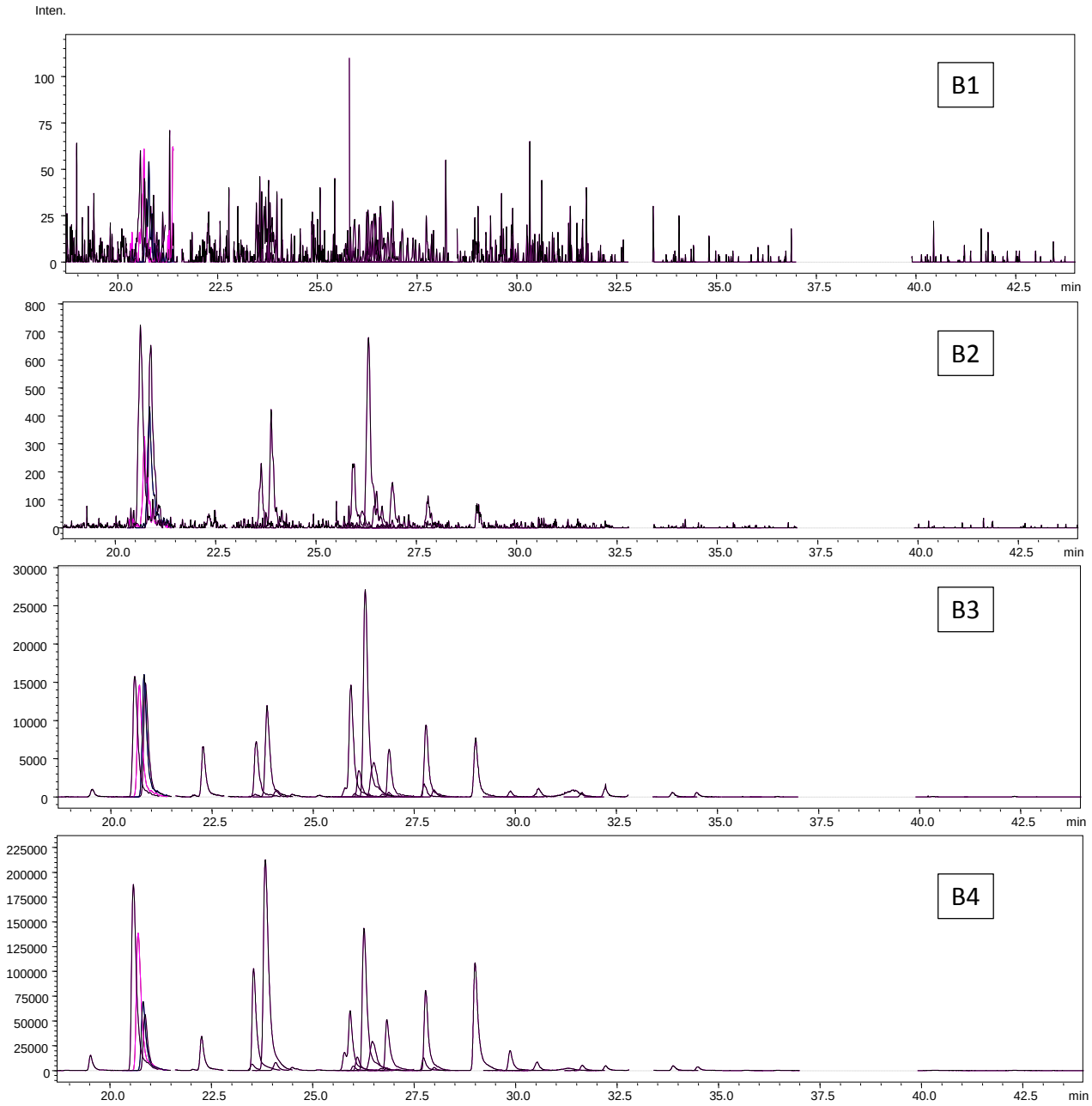


Figure 53 TIC chromatograms of MRM measurements of samples B1, B2, B3 and B4

Table 31 Peak areas determined in measurement of various samples A

Sample A1		Sample A2		Sample A3		Sample A3.2		Sample A4	
Event	Area	Event	Area	Event	Area	Event	Area	Event	Area
1	0	1	0	1	0	1	0	1	956
2	377	2	2771	2	10640	2	7516	2	20285
3	216	3	3002	3	10818	3	10283	3	19478
4	210	4	1178	4	4123	4	6063	4	12158
5	339	5	2244	5	13095	5	7118	5	21115
6	152	6	368	6	2314	6	2744	6	4377
7	124	7	737	7	3870	7	4878	7	9464
8	186	8	818	8	3942	8	3815	8	8883
9	0	9	0	9	255	9	356	9	753
10	144	10	1101	10	5092	10	6184	10	12661
11	0	11	0	11	0	11	0	11	174
12	0	12	187	12	1131	12	829	12	2050
13	384	13	1611	13	10275	13	9199	13	18758
14	126	14	466	14	3076	14	2201	14	4167
15	0	15	0	15	0	15	0	15	281
16	391	16	330	16	2788	16	2327	16	6559
17	0	17	0	17	0	17	0	17	128
18	0	18	0	18	0	18	0	18	0
19	0	19	0	19	434	19	279	19	976
20	0	20	340	20	1619	20	1573	20	4574
21	0	21	0	21	105	21	107	21	230
22	0	22	185	22	1162	22	2099	22	3792
23	0	23	0	23	0	23	0	23	0
24	0	24	0	24	359	24	707	24	644
25	0	25	0	25	0	25	0	25	0
26	0	26	0	26	0	26	216	26	95
27	0	27	0	27	0	27	117	27	154
28	0	28	0	28	0	28	0	28	0
29	0	29	0	29	0	29	0	29	0
30	0	30	0	30	0	30	0	30	0
31	0	31	0	31	0	31	0	31	0
32	0	32	0	32	0	32	0	32	0
33	0	33	0	33	0	33	0	33	0
SUM	2649	SUM	15338	SUM	75098	SUM	68611	SUM	152712

Table 32 Peak areas determined in measurement of various samples B

Sample B1		Sample B2		Sample B3		Sample B4	
Event	Area	Event	Area	Event	Area	Event	Area
1	0	1	0	1	7781	1	104541
2	418	2	6390	2	145983	2	1732298
3	322	3	3498	3	135248	3	605048
4	351	4	2443	4	133314	4	1115253
5	203	5	5596	5	132389	5	504158
6	0	6	389	6	50309	6	263903
7	346	7	1481	7	62100	7	853089
8	162	8	2734	8	103797	8	2062528
9	0	9	0	9	10172	9	95295
10	0	10	1839	10	128645	10	601310
11	0	11	0	11	3357	11	35568
12	0	12	477	12	28129	12	117897
13	0	13	5379	13	233190	13	1277022
14	0	14	1023	14	56298	14	379752
15	0	15	0	15	4334	15	20148
16	0	16	1191	16	49917	16	442207
17	0	17	0	17	1519	17	19694
18	0	18	0	18	865	18	11361
19	0	19	132	19	12915	19	101958
20	0	20	698	20	76564	20	660587
21	0	21	112	21	6517	21	21968
22	0	22	522	22	62146	22	948642
23	0	23	0	23	6105	23	167858
24	0	24	0	24	10516	24	71941
25	0	25	0	25	23836	25	49233
26	0	26	0	26	4341	26	45049
27	0	27	0	27	9159	27	106423
28	0	28	0	28	4400	28	38342
29	0	29	0	29	4390	29	31654
30	0	30	0	30	0	30	2999
31	0	31	0	31	243	31	2271
32	0	32	0	32	512	32	3878
33	0	33	0	33	460	33	2484
SUM	1802	SUM	33904	SUM	1509451	SUM	12496359

Sample B4 is the only conjugate, where all 33 MRM transitions could be detected in the measurement. It is also the highest modified protein conjugate, since the sum of peak areas in this measurement is the biggest. Peak areas were determined and summed up for quantification. The results are displayed in Table 31 and Table 32. We have chosen this approach for quantification to get relative information on modifications of peptides for each reaction sample. The sums of the detected peak areas were set in relation. This works only well if the peptides of interest are detected in both samples and the summed up peak areas of the same peptides are compared. For example if the 6 peaks detected in sample B1 would be set in relation to the total peak area determined in sample B4 (33 peaks), this would lead to a high deviation from the real result.

Since the ZEN-CMO-modification is distributed over the whole protein sequence and no peptide could be identified as the preferred site of modification, it did not seem meaningful to compare peak areas of single peptides. It cannot be concluded that one peptide is equally modified in all sample measurements. Therefore it seemed more reasonable to compare the sum of peak areas to get more information on the proteins and the modified peptides.

Peptides R.QEKR.F 3: ZEN-CMO (K) (MRM event 2) and K.TKCAR.N 2: ZEN-CMO (K), 3: CAM (C) (MRM event 3) were detected in all sample measurements (See Table 33).

Table 33 Peak area comparison of 2 single peptides R.QEKR.F 3: ZEN-CMO (K) (MRM event 2) and K.TKCAR.N 2: ZEN-CMO (K), 3: CAM (C) (MRM event 3) in ZEN-CMO-CON sample measurements

Sample measurement	Peak area event 2	Peak area event 3
A1	377	216
A2	2771	3002
A3	10640	10818
A4	20285	19478
B1	418	322
B2	6390	3498
B3	145983	135248
B4	1732298	605048

The two peptides show widely similar peak areas and are therefore easy to compare. While some peak area relations are closely the same, others are further apart. Peak area relation for

the samples A3:A4 is 1:1.9 determined in event 2 and 1:1.8 determined in event 3. However if samples B2:B3 are compared, event 2 gives a result of 1:22.8 and event 3 1:38.7. This occurs due to the fact, that not every peptide is equally modified in every ZEN-CMO-CON sample.

By comparing the sum of the peak areas of the peptides instead of single peptides, these disparities are expected to be evened out.

The main difference between samples A and B is the activation time. As shown in Figure 53 sample B1 was modified to a limited extent. In sample B2 modified peptides are detected. Sample A1 and B2 were synthesized with the same molar ratio for coupling (1:10), yet activation time was different. However B2 is modified to a greater extent than A1. Samples B show larger peak areas than samples A if the same cr was used during synthesis. This leads to the conclusion that longer activation time increases protein modification, without the need to increase the amount of ZEN-CMO for coupling.

The results for peak area relations are shown in Table 35. Previous to this study a MALDI-MS method was developed to quantify ZEN-CMO modifications (see C. Stephan[91]). The advantage of the MALDI-MS method is that the analytes are investigated on the intact protein level. Further it is possible to determine the average number of modifications per protein, while the LC-MS method can only be applied for relative quantification. In Table 34 results for MALDI-MS measurements are displayed. The number of average modifications per protein differs slightly because of different preparation methods.

Table 34 Results from MALDI-MS ZEN-CMO quantification. Unpublished data Sophie Fröhlich, 2013.

	Recalibrated (no ZipTip)		No ZipTip		ZipTip C_4 purification		Zip Tip C_4 Internal Calibration	
n = 10	Δ M/M_h	rounded	Δ M/M_h	rounded	Δ M/M_h	rounded	Δ M/M_h	rounded
A_1 (1:10)	-0.6	-1	-0.6	-1	0.2	0	0.0	0
A_2 (1:25)	-0.1	0	-0.5	0	0.5	0	0.2	0
A_3 (1:50)	0.8	1	1.0	1	1.7	2	1.3	1
A_4 (1:75)	2.8	3	2.1	2	2.9	3	2.8	3
B_1 (1:5)	-0.7	-1	-0.6	-1	0.1	0	-0.1	0
B_2 (1:10)	0.0	0	-0.2	0	0.8	1	0.5	1
B_3 (1:25)	4.3	4	3.3	3	4.6	5	4.1	4
B_4 (1:50)	20.5	20	15.9	16	x	x	0.3/14.0	0/14

A selection of results for relative quantification via LC-MS/MS measurements is shown in Table 35. The summed up peak areas from each measurement were set in relation. Results are compared to ratios of modification density, determined by MALDI-MS measurements with ZipTip purification, for comparison. MALDI-MS ratios were calculated based on unpublished data by Sophie Fröhlich, 2013.

Table 35 Results for relative quantification by LC-MS/MS. Ratios of modification densities for different samples determined by MALDI-MS and LC-MS/MS are shown in comparison. - = no calculation possible

Samples	LC-MS/MS Peak area relation	MALDI MS ZipTip C_4 purification	MALDI MS ZipTip C_4 Internal Calibration
A1 : A2	1 : 5.5	1 : 2.5	-0 : 0.2
A1 : B1	1 : 1.2	1 : 2.0	0:-0.1
B1 : B2	1 : 12.3	1 : 8.0	-0.1:0.5
A2 : A3	1 : 4.8	1 : 3.4	1 : 6.5
A3 : A4	1 : 2.0	1 : 1.7	1 : 2.2
B2 : B3	1 : 41.8	1 : 5.8	1 : 8.2
B3 : B4	1 : 8.3	-	-
A2 : B2	1 : 2.2	1 : 1.6	1 : 2.5
B2 : A3	1 : 2.2	1 : 2.1	1 : 2.6
B2 : A4	1 : 4.4	1 : 3.6	1 : 5.6
A3 : B3	1 : 19.2	1 : 2.7	1 : 3.2
A4 : B3	1 : 9.6	1 : 1.6	1 : 1.5
A3 : B4	1 : 157.9	-	-

Samples A and B differ by activation time and coupling ratios (See Table 3). Since sample A1 and B1 were determined to be modified to a very low extent (on average no modification), comparison of relative quantification results is not significant, however listed in the table for completeness. Peak areas from LC-MS/MS measurements were also very small, indicating also a very low degree of modification for these samples. In sample A1 more peaks were detected than in B1, however the sum of the detected peak areas in B1 exceed those of A1. Events 2,3,4,5 and 8 showed small peaks in both measurements. Events 6,10,13,14 and 16 were additionally detected in sample A1. However the peak areas are very small and were considered as noise and not peptides. The same peptides were also detected in all samples with a higher degree of modification. LC-MS/MS results for samples A1, A2, A3, A4, B1 and B2 are in line with the results from MALDI-MS after ZipTip purification. Since the amount of ZEN-CMO

modifications is not identical for each protein and conjugate synthesis is not 100% reproducible, the results are never exactly the same.

The results for samples B3 and B4 determined by LC-MS/MS were however not matching the results from MALDI-MS measurements. In both cases B3 and B4 are the highest modified samples, by LC-MS/MS however an even higher grade of modification was determined than by MALDI-MS. It remains undetermined, which method achieves more accurate results. Such a high amount of modification for samples B3 and B4 as determined by LC-MS/MS is however very unlikely due to the number of possible modification sites on the protein. Measurement results might have been influenced by peptide concentration.

Of course there is still room for improvement for the developed method. At the moment, only peptides observed in the preceeding nLC-nESI-QIT experiments were measured on the QQQ. Which means that only 32 out of 96 possible peptides were monitored (max. 2 missed cleavages and modified K). The doubly and triply charged peptide was only measured for sequence R.KDSNVNWNNLKGK.K, since both could be identified in previous measurements. All other peptides were measured either as doubly or triply charged, according to which ion was identified in previous measurements. The method could be expanded by measuring all possible modification sites, e.g. all peptides containing Lys, and more charge states for higher accuracy.

Further this method is influenced by sample concentration. If the sample concentration is not the same for the compared samples or if protein is lost during sample preparation, this influences the results. Therefore sample preparation has to be carried out very carefully. This problem could be avoided by using an internal standard for the complete method.

Problems concerning the quantification method

To further develop the LC-MS/MS method, a standard for quantification needs to be chosen. None of the samples can be used as standard, since the conjugate synthesis does not always result in an equal amount of modifications per protein. Therefore a standard conjugate might differ in amount of ZEN-CMO modifications and a newly synthesized standard might produce different results. The result accuracy is therefore depending on the synthesis of the conjugate standard.

Because of that no absolute numbers for protein modification can be determined. This is however a necessity for Romer Labs. Therefore a different approach was tested.

3.5.6 Approach to determine the grade of modification

Since no statement about the grade of modification can be made, in terms of absolute numbers, and a ZEN-CMO-CON sample is unreliable to use as a standard, the relative quantification method is not satisfying.

Therefore another approach was chosen, which would allow to determine the grade of modification (GOM) by comparing peak areas (See Equ. 3.1).

$$GOM = \frac{A_{MOD}}{A_{MOD} + A_{UNMOD}} \quad [3.1]$$

Modified as well as the corresponding unmodified peptides are measured in the same sample measurement. By setting the peak areas of the modified peptides in relation to the sum of modified plus unmodified peptides, a certain coefficient can be determined, which describes the grade of modification. For this it was first necessary to identify the unmodified peptides to the corresponding modified ones. Yet it has to be mentioned that it was not possible to identify these unmodified peptides in previous measurements on the nLC-nESI-QIT. As experiments cannot be transferred between different instruments without checking results in detail, especially when comparing nLC and UPLC as separation systems and QIT and QQQ as mass analyzers, SIM experiments were performed on the UPLC-QQQ system to check for the registration of all possible unmodified peptides detected in their doubly and triply charged form. This experiment will allow to identify the RT of the unmodified peptides. All peptides were measured as doubly as well as triply charged in the same measurement. A peptide should lead to the formation of a peak at the same RT in both chromatograms, allowing conclusions on the peptides RT.

In the results not all peptides were detected as doubly as well as triply charged ions. Almost all chromatograms showed more than one peak, not allowing clear determination of the peptides RT.

Therefore RTs from nLC measurements were investigated for further information. Since not all peptides were identified in measurements using gradient B, theoretical nLC-RTs determined by “TheorChromo” were taken into account. As already shown in chapter 3.4 RT on UPLC is 31.2 min (± 1.4 min) less compared to nLC measurements using gradient B. This information was also taken into account to set the time frames for the next measurements.

The next step was to identify proper product ions for the unmodified peptides to define MRM transitions. Therefore MS/MS spectra of the corresponding unmodified peptides, which were

identified on the QIT were investigated and the most intense fragment ions were chosen as product ions for MRM. The doubly, as well as the triply charged peptides were used as precursor ions. CON was digested using trypsin/LysC and desalted using C_{18} spin columns. 78 pmol were measured. Settings for the first measurement are displayed in Table 16 in chapter 2.5.4. Each peptide was measured in its doubly as well as triply charged state with both product ions. The result is shown in Figure 54. Only peptide R.SAGWNIPIGTLLHR.G was detected with a very intense signal. Peptides R.IQWCAVGKDEK.S and K.KGTEFTVNDLQGK.T were detected as well. No other peptide was detected.

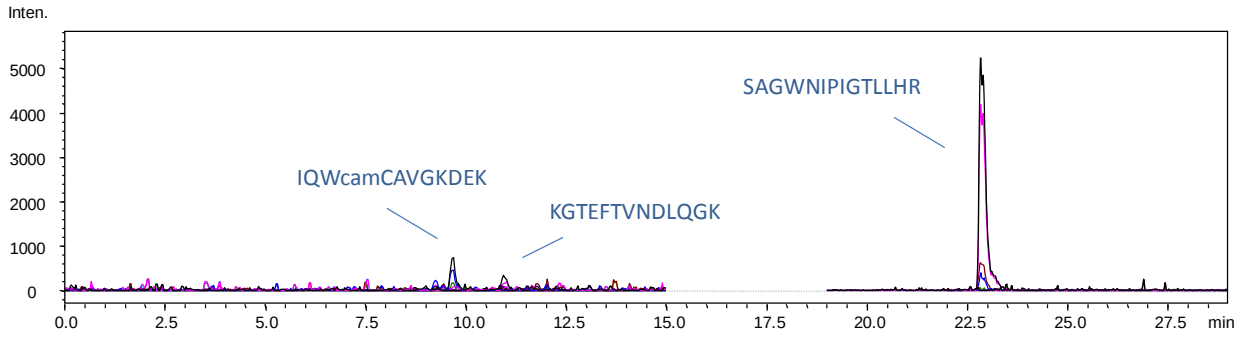


Figure 54 TIC chromatogram of unmodified peptides of CON, 78 pmol, -30 V

Since so many MRM transitions were measured in the same time frame, it is possible that the instrument's dwell time was inappropriate to detect all peptides of interest (limit of detection not low enough). Therefore the experiment was repeated, however the absolute amount of peptide was increased to 300 pmol and less MRM transitions per time frame were measured. Measurement settings are listed in Table 17 in chapter 2.5.4.

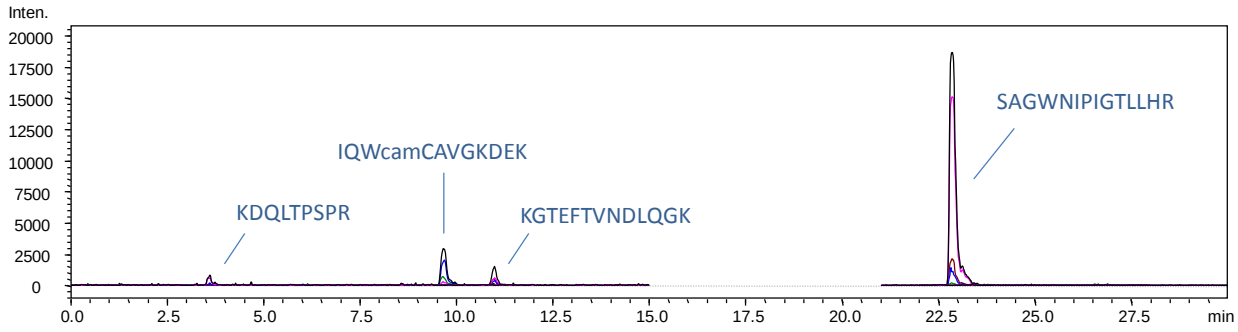


Figure 55 TIC chromatogram of unmodified peptides of CON, 300 pmol, -30 V

Peptide R.SAGWNIPIGTLLHR.G could be detected with high intensity. Also 3 more peptides were detected (see Figure 55). These peptides could also be identified in QIT measurements. The increase in peak areas correlates roughly with the increase in amount of peptide. The

increase in injection amount seems reasonable, since peak areas increased and are easier to detect. Also one more peak could be detected compared to the previous measurement. It remains unknown at this point why the other peptides could not be detected. Possible explanations are given below.

An important point for peak detection is that MRM transitions have to be well chosen. For the detection of a peptide, it has to be determined, if the peptide occurs as doubly or triply charged. Also not all possible product ions are suitable for MRM, as shown in Figure 56.

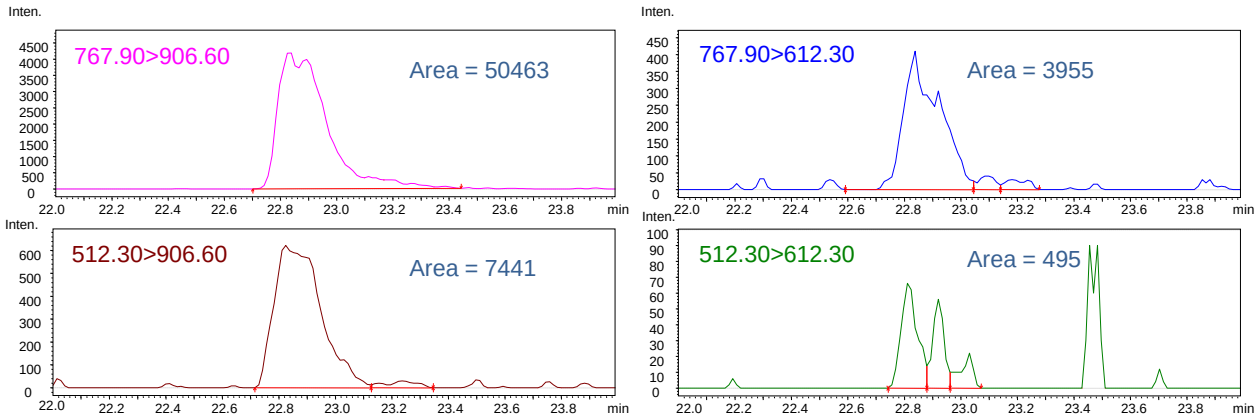


Figure 56 MRM transitions for the doubly (m/z 767.9) and triply (m/z 512.3) charged peptide R.SAGWNIPIGTLLHR.G with product ions y_8 (m/z 906.6) and b_{12} (m/z 612.3). CE: -30 V

In Figure 56 four MRM transitions for peptide R.SAGWNIPIGTLLHR.G are displayed. Based on previous experiments (see chapter 3.5.2) the CE of -30 V was applied in all measurements and the effect of the chosen precursor and fragment ion on peak areas was observed. It can be observed that the different transitions result in different intensities and peak areas in the chromatogram. While transition of the doubly charged peptide and product ion y_8^{1+} can be measured with a peak area of 50463, the triply charged peptide forming the product ion b_{12}^{2+} results in a peak area of only 495. Thus it is inevitable to find ideal MRM transitions for the peptides. For these experiments product ions were selected from the most intense fragment ion peaks from QIT measurement results. The application of CE differs between a QIT and a QQQ, therefore the product ions might not be ideal. Also peptide fragmentation is dependent on accurate fragmentation energy, so ideally the fragmentation energy should be adapted for each peptide to form intense product ions. If these parameters are not well chosen, the peptide will not be detected. The chosen MRM transitions might not be ideal in these measurements.

For this it can be said that further work has to include the optimization of CE for each peptide and possible transition. Using software packages like “Skyline”[99] can help as an automated workflow can be defined.

Another problem is that many small, per se not very hydrophobic peptides were detected only as their modified analogue. These are also the peptides, which ionize best as modified and are best detected in the modified samples. The unmodified peptides are not very hydrophobic and possibly not retained by the C_{18} column, so they might elute in the flow through and cannot be separated. It was not searched for these peptides in this experiment.

Also the modification might pose a steric hindrance for protein digestion, since Lys are preferably modified. Therefore the peptides were not cleaved at the modified Lys and the modified peptides have more missed cleavages. The unmodified peptides were detected with less missed cleavages in QIT measurements. Therefore different peptides may form, depending on if the protein is modified or not (See Table 36). MASCOT searches were however performed by searching for peptides with maximum one missed cleavage, therefore it cannot be said with certainty that peptides with more missed cleavages are not also produced in a digestion of unmodified proteins.

Table 36 ZEN-CMO modified peptide with missed cleavages at the modified AA and matching unmodified peptides without the missed cleavages

Modified peptides	Unmodified peptides
K.SDFHLFGPPGKK.D 11: ZEN-CMO (K)	**K.SDFHLFGPPGK.K**
R.KDSNVNWNNLKGK.K 11:ZEN-CMO (K)	**R.KDSNVNWNNLK.G**
K.DSAIMLKR.V 7: ZEN-CMO (K)	**K.DSAIMLK.R**
K.DLTKCLFK.V 4: ZEN-CMO (K), 5: CAM (C)	**K.DLLFKDLTK.C**
R.FGVNGSEKSK.F 8: ZEN-CMO (K)	**R.FGVNGSEK.S**
K.SDFHLFGPPGKKDPVLK.D 11:ZEN-CMO (K)	**K.SDFHLFGPPGK.K**
R.KDSNVNWNNLK.G 1:ZEN-CMO (K)	**K.DSNVNWNNLK.G**
R.IQWCAVGKDEK.S 4: CAM (C), 8: ZEN-CMO (K)	**R.IQWCAVGK.D** 4: CAM (C)

R.WCTISSPEEKK.C 2: CAM (C), 10: ZEN-CMO (K)	**R.WCTISSPEEK.C** 2: CAM (C)
K.SKFMMFESQNK.D 2: ZEN-CMO (K)	**K.FMMFESQNK.D**
K.DSNVNWNNLKGK.K 10: ZEN-CMO (K)	**K.DSNVNWNNLK.G**
K.KGTEFTVNDLQGK.T 1: ZEN-CMO (K)	**K.GTEFTVNDLQGK.T**
R.EGTTYKEFLGDKFYTVISSLK.T 12: ZEN-CMO (K)	**R.EGTTYKEFLGDK.F**
K.EFLGDKFYTVISSLK.T 6: ZEN-CMO (K)	**K.FYTVISSLK.T**
K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K)	**K.FMMFESQNK.D**

Time frames were set for the measurements. The exact RT for the unmodified peptides is not known. RT might have been estimated wrongly, which could be another reason why the peptides were not detected.

3.5.7 Comparison of ionization efficiencies of a ZEN-CMO modified and an unmodified peptide

Further it was of interest, whether ZEN-CMO modification would influence a peptide´s ionization efficiency. Therefore an equimolar mix of the ZEN-CMO modified and the unmodified test peptide KRTLRR (121 pmol) was prepared and measured in a Q1 scan after separation. Peak areas for the doubly charged peptides were compared. The doubly charged modified peptide corresponds to the m/z value 602, the unmodified peptide to m/z 415.

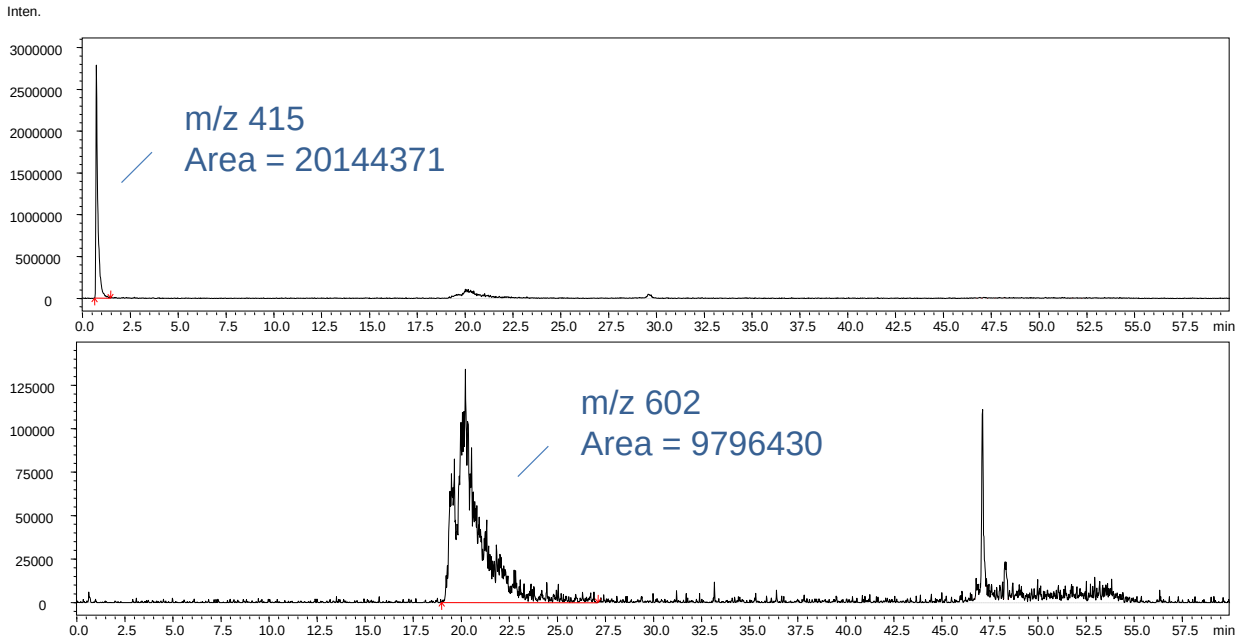


Figure 57 Comparison of peak areas of the doubly charged modified peptide (m/z 602) and the doubly charged unmodified peptide (m/z 415) KRTLRR. The unmodified peptide is displayed on the top. The modified peptide is displayed on the bottom.

Extracted ion chromatograms for m/z 602 and m/z 415 are shown in Figure 57. Peak area ratio of modified : unmodified peptide is 1 : 2.1 This would lead to the conclusion that the ionization efficiency for the unmodified, more hydrophilic peptide is higher than for the more hydrophobic ZEN-CMO modified peptide.

However it has to be mentioned that the LC gradient can influence peptide ionization efficiency, since some peptides might ionize better at a low percentage of ACN. Unfortunately the results are not reliable. ZEN-CMO modification of the peptide was not complete. The modified peptide sample contains unmodified peptide as well. A peak at m/z 415 is visible in the Q1 scan of this sample. This adds to the peak area. Further it is unknown, if all peptide was preserved after modification. Therefore the mixture might not be equimolar and the results have to be considered doubtful.

4 Conclusion

The ZEN quick test is a useful tool to determine mycotoxin contaminations in corn samples. ZEN-CMO-CON conjugates are an essential compound in this test (see chapter 1.2). Improvement in the conjugate synthesis method is however still desired to reduce reaction times and costs. A LC-MS/MS method for quantifying ZEN-CMO modifications on CON was of interest to determine the amount of modifications introduced onto a protein by certain synthesis strategies.

To optimize protein digestion two different enzymes for digestion were tested. Trypsin was compared to a mix of trypsin and LysC. Trypsin/LysC mix gave better results in terms of SC. Since a high SC was important to characterize the protein and to identify possible ZEN-CMO modification sites, trypsin/LysC mix was used for further experiments.

ZEN-CMO-CON conjugates were characterized by nLC-nESI-QIT. It was possible to identify certain ZEN-CMO modification sites on CON. In total 28 modified AAs could be identified. While Lys was identified as the preferred AA residue for coupling, modification is also possible for Ser. The modified AAs were mainly identified on the proteins surface, which is reasonable, for a protein in its globular form as the surface is easily accessed for ligation.

By investigating peptide RTs of the unmodified and the modified peptides, it could be observed that ZEN-CMO modification increases peptide´s hydrophobicity significantly showing how hydrophobic this modification is.

After the protein and its modification sites were characterized on the nLC-nESI-QIT the gained information was used to develop a LC-MS/MS method for quantification. Since the QIT has a limited dynamic range, the quantification method was developed on an UPLC-ESI-QQQ instrument. Also a QQQ allows for targeted analysis. Precursor ion scan, product ion scan, neutral loss scan and MRM are possible scan modes on a QQQ. Fragment ions generated by low-energy CID experiments, as performed in QITs or QQQ instruments were of interest for the method. Therefore the fragmentation pattern of a ZEN-CMO modified peptide was important. Thus a peptide was ZEN-CMO modified and measured with product ion scan to identify possible reporter ions. Since it could be observed that peptide charge influences fragmentation behavior, different fragmentation energies were tested. A fragmentation energy of -30 V was chosen, since it was suitable to fragment doubly as well as triply charged peptides. Just as in QIT measurements an intense fragment ion of m/z 203 could be observed in QQQ measurements

also. Due to its high intensity and specificity this fragment ion was chosen for product ion scan and MRM.

Neutral loss scan, precursor ion scan and MRM were tested as possible scan modes for the quantification method. Neutral loss scan was excluded as method of choice, since no reasonable peaks were detected. The reason for this can be badly adapted fragmentation energies or a sample amount below the limit of detection. Precursor ion scan met the same limitations. Due to the noise registered for the chromatogram, the modified peptides could only be detected in samples with high cr.

Further it is of notice that m/z 203 is not specific enough to uniquely identify ZEN-CMO modified peptides, since some peptides in CON produce fragment ions of m/z 203. Therefore MRM was chosen as the best suitable method. Noise is reduced to a minimum - if not zero if time frames are defined for MRM transitions - and very sensitive detection is possible. Yet we found that in the samples with low cr not all ZEN-CMO modified peptides were detected. A quantification approach is possible with a lower number of detected peaks. Detecting more peptides would however increase the significance of results. We found that increased activation time allows for reducing the molar cr. The number of ZEN-CMO modification appears to be maintained.

Although the developed MRM method seems to work, the method has its drawbacks:

- Use of a standard for quantification: For the final method it was contemplated to use a ZEN-CMO-CON conjugate as standard. Unfortunately the production of the conjugates is not 100% reproducible. Thus a newly produced conjugate might have a different ZEN-CMO density and thereby the results for quantification would be different.
- Peptide concentration: Further the method is influenced by peptide concentrations, which might also lead to inaccurate results.
- No measurement of the whole protein: Only the identified possibly modified peptides are measured in this method and not the complete protein is monitored.
- False positive results: The fragment at m/z 203 can also be produced by unmodified peptides. Even though false positive results are unlikely due to RTs, they may still occur.
- No absolute quantification: The method does not give an average number of modifications per protein and allows only relative quantification.

Because of these problems a different approach for quantification was tested. Contrary to the first method, the determination of the grade of modification should be possible with this alternative method. For this, the unmodified and the modified peptides have to be detected in the

same UPLC-QQQ measurement. From the resulting integration areas for the individual peptides, modified and unmodified, the grade of modification can be deduced. Since the modified peptides were already identified, detection of the corresponding unmodified peptides was needed.

However it was not possible to reasonably detect the unmodified peptides corresponding to the modified ones. Many of the unmodified peptides are not very hydrophobic and thereby possibly not retained by the RPC column. Further it is very likely that the ZEN-CMO-CON modification obstructs protein digestion and that different peptides are formed from a conjugate than from unmodified CON. Additionally the chosen MRM transitions might not be ideal, since fragment ions were chosen according to QIT data and fragmentation behavior in a QQQ can be different. For ideal MRM it has to be determined, if a peptide is doubly or triply charged and which fragment ions are formed with a high intensity. For the best results the CEs should be adjusted for every peptide. The development of the second quantification method would require more time and further studies are required.

5 Outlook

The method developed in this study is only usable for relative quantification. Therefore a method for absolute quantification is still desired. In this thesis some experiments were already conducted to develop a method to determine the grade of modification. Unfortunately they were not very successful. To develop the method further, suitable MRM transitions are still required. Peptide charge has to be determined as well as suitable product ions. Further the fragmentation energy should be adapted for each peptide and RT has to be determined.

"Skyline" is an open source software, which can be used to create targeted proteomic experiments. Via "Skyline" it is possible to predict RTs for peptides as well as CEs.[99,100] By using this software MRM transitions for unmodified peptides could be determined and optimized MRM transitions for the unmodified peptides could be created.

Besides ZEN there are many other mycotoxins that are regular contaminants of crops worldwide. Quick tests for other (more costly) mycotoxins – for example aflatoxin, vomitoxin and fumonisin – have already been developed by Romer Labs. Especially aflatoxin is rather expensive and a low-cost production for an aflatoxin quick test is desired. The developed LC-MS/MS quantification method is only applicable for ZEN-CMO modified CON. For the quantification of other modifications, modified AA residues and fragment ions for MRM have to be newly identified. This is not required for the MALDI-MS method by C. Stephan.[91] MALDI-MS is therefore the superior method.

6 References

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7 Appendix

7.1 Mascot search result details for “Chain A, Crystal Structure of Aluminium-Bound Ovotransferrin At 2.15 Angstrom Resolution” for comparison of trypsin and trypsin/LysC mix for protein digestion

Immobilized trypsin, digestion 1, measurement 1:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	874
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 34%

Matched peptides shown in ***bold red***.

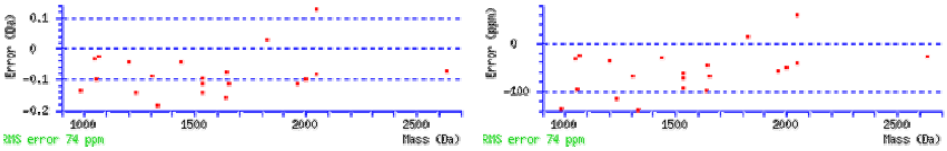
```
   1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
  51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
 101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
 151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
 201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
 251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
 301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
 351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
 401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
 451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
 501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
 551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
 601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
 651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
78	9 – 18	618.7100	1235.4054	1235.5492	-0.1438	0	42	13	1		R.WCTISSPEEK.K
44	40 – 47	492.1800	982.3454	982.4794	-0.1339	0	25	4.1e+002	2		K.ATYLDCIK.A
249	48 – 73	1317.6200	2633.2254	2633.2973	-0.0718	0	99	1.4e-005	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
42	100 – 112	479.5700	1435.6882	1435.7307	-0.0425	1	40	14	2		K.KGTEFTVNDLQGK.T
81	101 – 112	654.7800	1307.5454	1307.6358	-0.0903	0	58	0.27	1		K.GTEFTVNDLQGK.T
48	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	32	78	1		R.SAGWNIPIGTLIHR.G
49	122 – 135	512.2500	1533.7282	1533.8416	-0.1134	0	19	1.7e+003	10		R.SAGWNIPIGTLIHR.G
144	122 – 135	767.8800	1533.7454	1533.8416	-0.0961	0	47	2.9	1		R.SAGWNIPIGTLIHR.G
222	136 – 154	980.4300	1958.8454	1958.9585	-0.1131	0	81	0.0013	1		R.GAIEWEGIESGSVEQAVAK.F
170	155 – 169	821.3200	1640.6254	1640.7869	-0.1614	0	54	0.72	1		K.FFSASCVPGATIEQK.L
59	270 – 279	534.2400	1066.4654	1066.4931	-0.0277	0	44	5.6	1		K.AQSDFGVDTK.S
29	280 – 290	401.1900	1200.5482	1200.5928	-0.0446	0	30	1.8e+002	1		K.SDFHLFGPPGK.K
61	424 – 438	551.5800	1651.7182	1651.8318	-0.1136	0	59	0.21	1		K.TDERPASYFAVAVAR.K
37	439 – 449	444.5000	1330.4782	1330.6629	-0.1848	1	20	1.9e+003	10		R.KDSNVNWNNLK.G
172	498 – 512	822.3600	1642.7054	1642.7807	-0.0753	0	33	76	1		R.LCQLCQGSGGIPPEK.C
55	521 – 529	524.2500	1046.4854	1046.5185	-0.0331	0	37	27	1		K.YFGYTGALR.C
209	560 – 574	913.9200	1825.8254	1825.7975	0.0280	0	68	0.026	1		K.NLQMDDFELLCTDGR.R
86	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	30	1.5e+002	3		R.ECNLAEVPTHAVVVRPEK.A
88	583 – 600	683.4000	2047.1782	2047.0520	0.1261	0	24	6.1e+002	9		R.ECNLAEVPTHAVVVRPEK.A
56	661 – 669	529.2500	1056.4854	1056.5855	-0.1001	0	37	29	1		K.FYTVISSLK.T
226	670 – 686	1000.4000	1998.7854	1998.8849	-0.0995	0	72	0.0098	1		K.TCNPSDILQMCSFLEGK.-



Immobilized trypsin, digestion 1, measurement 2:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database: NCBInr
Score: 928
Nominal mass (M_r): 77518
Calculated pI: 6.70
Taxonomy: Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file: DATA.TXT
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 34%

Matched peptides shown in ***bold red***.

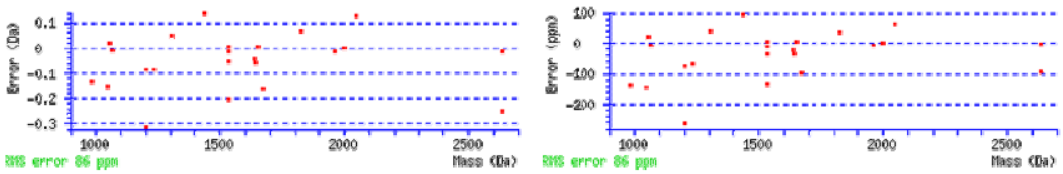
```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
81	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	43	11	1		R.WCTISSPEEK.K
46	40 – 47	492.1800	982.3454	982.4794	-0.1339	0	30	1.4e+002	3		K.ATYLDCIK.A
185	48 – 73	878.6900	2633.0482	2633.2973	-0.2491	0	20	1.8e+003	2	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
250	48 – 73	1317.6500	2633.2854	2633.2973	-0.0118	0	118	2e-007	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
44	100 – 112	479.6300	1435.8682	1435.7307	0.1375	1	41	12	1		K.KGTEFTVNDLQGK.T
86	101 – 112	654.8500	1307.6854	1307.6358	0.0497	0	71	0.013	1		K.GTEFTVNDLQGK.T
50	122 – 135	512.2200	1533.6382	1533.8416	-0.2034	0	48	2	1		R.SAGWNIPIGTLIHR.G
51	122 – 135	512.2700	1533.7882	1533.8416	-0.0534	0	60	0.14	1		R.SAGWNIPIGTLIHR.G
149	122 – 135	767.9200	1533.8254	1533.8416	-0.0161	0	33	74	6		R.SAGWNIPIGTLIHR.G
150	122 – 135	767.9300	1533.8454	1533.8416	0.0039	0	39	20	1		R.SAGWNIPIGTLIHR.G
212	136 – 154	980.4800	1958.9454	1958.9585	-0.0131	0	94	6.1e-005	1		R.GAIEWEGIESGSVEQAVAK.F
164	155 – 169	821.3800	1640.7454	1640.7869	-0.0414	0	76	0.0049	1		K.FFSASCVPGATIEQK.L
68	185 – 199	557.8700	1670.5882	1670.7511	-0.1629	0	14	6.6e+003	9		R.NAPYSGYSGAFHCLK.D
59	270 – 279	534.2500	1066.4854	1066.4931	-0.0077	0	41	12	1		K.AQSDFGVDTK.S
32	280 – 290	401.1000	1200.2782	1200.5928	-0.3146	0	27	3.5e+002	4		K.SDFHLFGPPGK.K
77	280 – 290	601.2600	1200.5054	1200.5928	-0.0873	0	25	7.2e+002	9		K.SDFHLFGPPGK.K
62	424 – 438	551.6200	1651.8382	1651.8318	0.0064	0	48	2.2	1		K.TDERPASYFAVAVAR.K
167	498 – 512	822.3700	1642.7254	1642.7807	-0.0553	0	33	92	1		R.LCQLCQGSGGIPPEK.C
55	521 – 529	524.1900	1046.3654	1046.5185	-0.1531	0	32	84	1		K.YFGYTGALR.C
195	560 – 574	913.9400	1825.8654	1825.7975	0.0680	0	55	0.46	1		K.NLQMDDFELLCTDGR.R
94	583 – 600	683.4000	2047.1782	2047.0520	0.1261	0	32	1.1e+002	1		R.ECNLAEVPTHAVVVRPEK.A
56	661 – 669	529.3100	1056.6054	1056.5855	0.0199	0	27	2.8e+002	1		K.FYTVISSLK.T
217	670 – 686	1000.4500	1998.8854	1998.8849	0.0005	0	89	0.00019	1		K.TCNPSDILQMCSFLEGK.-



Immobilized trypsin, digestion 1, measurement 3:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database: NCBInr
Score: 797
Nominal mass (M,): 77518
Calculated pI: 6.70
Taxonomy: Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file: DATA.TXT
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 34%

Matched peptides shown in ***bold red***.

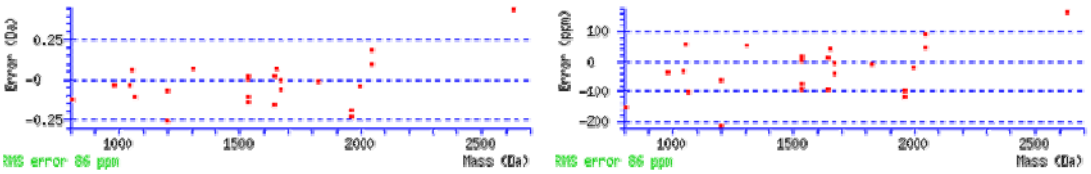
```
   1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
  51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
 101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
 151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
 201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
 251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
 301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
 351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
 401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
 451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
 501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
 551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
 601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
 651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
39	40 – 47	492.2300	982.4454	982.4794	-0.0339	0	25	4.2e+002	2		K.ATYLDCIK.A
180	48 – 73	878.9200	2633.7382	2633.2973	0.4409	0	41	13	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
77	101 – 112	654.8600	1307.7054	1307.6358	0.0697	0	86	0.00038	1		K.GTEFTVNDLQGK.T
43	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	32	78	1		R.SAGWNIPIGTLIHR.G
44	122 – 135	512.2500	1533.7282	1533.8416	-0.1134	0	36	30	1		R.SAGWNIPIGTLIHR.G
45	122 – 135	512.2900	1533.8482	1533.8416	0.0066	0	52	0.87	1		R.SAGWNIPIGTLIHR.G
139	122 – 135	767.9400	1533.8654	1533.8416	0.0239	0	35	47	1		R.SAGWNIPIGTLIHR.G
206	136 – 154	980.3700	1958.7254	1958.9585	-0.2331	0	39	18	1		R.GAIEWEGIESGSVEQAVAK.F
207	136 – 154	980.3900	1958.7654	1958.9585	-0.1931	0	77	0.003	1		R.GAIEWEGIESGSVEQAVAK.F
154	155 – 169	821.4100	1640.8054	1640.7869	0.0186	0	55	0.58	1		K.FFSASCVPGATIEQK.L
164	185 – 199	836.3500	1670.6854	1670.7511	-0.0657	0	38	29	1		R.NAPYSGYSGAFHCLK.D
165	185 – 199	836.3800	1670.7454	1670.7511	-0.0057	0	21	1.5e+003	2		R.NAPYSGYSGAFHCLK.D
32	241 – 246	404.1200	806.2254	806.3493	-0.1239	0	33	78	8		K.TCNWAR.V
52	270 – 279	534.2000	1066.3854	1066.4931	-0.1077	0	33	62	1		K.AQSDFGVDTK.S
27	280 – 290	401.1200	1200.3382	1200.5928	-0.2546	0	25	5.6e+002	1		K.SDFHLFGPPGK.K
28	280 – 290	401.1800	1200.5182	1200.5928	-0.0746	0	29	2.4e+002	1		K.SDFHLFGPPGK.K
55	424 – 438	551.6400	1651.8982	1651.8318	0.0664	0	56	0.39	1		K.TDERPASYFAVAVAR.K
156	498 – 512	822.3200	1642.6254	1642.7807	-0.1553	0	37	33	1		R.LCQLCQGSGGIPPEK.C
49	521 – 529	524.2500	1046.4854	1046.5185	-0.0331	0	34	55	1		K.YFGYTGALR.C
190	560 – 574	913.9000	1825.7854	1825.7975	-0.0120	0	64	0.066	1		K.NLQMDDFELLCTDGR.R
82	583 – 600	683.3900	2047.1482	2047.0520	0.0961	0	25	4.7e+002	10		R.ECNLAEVPTHAVVVRPEK.A
83	583 – 600	683.4200	2047.2382	2047.0520	0.1861	0	32	97	3		R.ECNLAEVPTHAVVVRPEK.A
50	661 – 669	529.3300	1056.6454	1056.5855	0.0599	0	38	26	1		K.FYTVISSLK.T
210	670 – 686	1000.4300	1998.8454	1998.8849	-0.0395	0	70	0.014	1		K.TCNPSDILQMCSFLEGK.-



Immobilized trypsin, digestion 2, measurement 1:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database: NCBInr
Score: 775
Nominal mass (M_r): 77518
Calculated pI: 6.70
Taxonomy: Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file: DATA.TXT
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 32%

Matched peptides shown in ***bold red***.

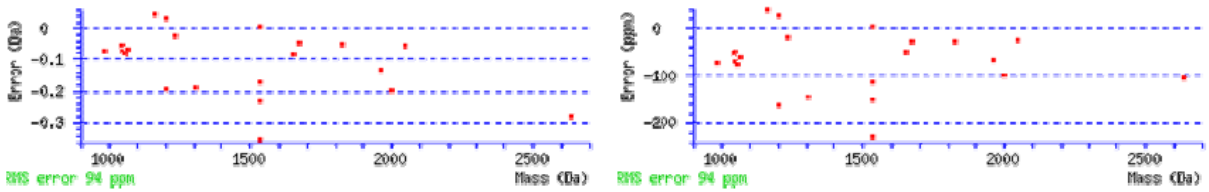
```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
79	9 – 18	618.7700	1235.5254	1235.5492	-0.0238	0	31	2.1e+002	1		R.WCTISSPEEK.K
46	40 – 47	492.2100	982.4054	982.4794	-0.0739	0	28	2.3e+002	2		K.ATYLDCIK.A
197	48 – 73	878.6800	2633.0182	2633.2973	-0.2791	0	32	1.1e+002	1	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
84	101 – 112	654.7300	1307.4454	1307.6358	-0.1903	0	67	0.03	1		K.GTEFTVNDLQGK.T
49	122 – 135	512.1700	1533.4882	1533.8416	-0.3534	0	33	72	1	U	R.SAGWNIPIGTLIHR.G
50	122 – 135	512.2100	1533.6082	1533.8416	-0.2334	0	34	49	1	U	R.SAGWNIPIGTLIHR.G
51	122 – 135	512.2300	1533.6682	1533.8416	-0.1734	0	32	92	1	U	R.SAGWNIPIGTLIHR.G
150	122 – 135	767.9300	1533.8454	1533.8416	0.0039	0	40	17	2	U	R.SAGWNIPIGTLIHR.G
222	136 – 154	980.4200	1958.8254	1958.9585	-0.1331	0	80	0.0013	1		R.GAIEWEGIESGSVEQAVAK.F
182	185 – 199	836.3600	1670.7054	1670.7511	-0.0457	0	42	12	1		R.NAPYSGYSGAFHCLK.D
59	270 – 279	534.2200	1066.4254	1066.4931	-0.0677	0	42	8.6	1		K.AQSDFGVDTK.S
31	280 – 290	401.1400	1200.3982	1200.5928	-0.1946	0	33	87	1		K.SDFHLFGPPGK.K
74	280 – 290	601.3200	1200.6254	1200.5928	0.0327	0	23	1e+003	2		K.SDFHLFGPPGK.K
55	333 – 341	521.2600	1040.5054	1040.5614	-0.0560	1	39	19	1		R.KDQLTPSPR.E
62	424 – 438	551.5900	1651.7482	1651.8318	-0.0836	0	57	0.3	1		K.TDERPASYFAVAVAR.K
56	521 – 529	524.2300	1046.4454	1046.5185	-0.0731	0	35	49	1		K.YFGYTGALR.C
57	521 – 529	524.2400	1046.4654	1046.5185	-0.0531	0	36	38	1		K.YFGYTGALR.C
212	560 – 574	913.8800	1825.7454	1825.7975	-0.0520	0	73	0.0076	1		K.NLQMDDFELLCTDGR.R
94	583 – 600	683.3400	2046.9982	2047.0520	-0.0539	0	36	42	1		R.ECNLAEVPTHAVVVRPEK.A
69	625 – 633	581.2800	1160.5454	1160.4994	0.0460	0	18	2.5e+003	2		K.FMMFESQNK.D
58	661 – 669	529.2600	1056.5054	1056.5855	-0.0801	0	30	1.6e+002	1		K.FYTVISSLK.T
226	670 – 686	1000.3500	1998.6854	1998.8849	-0.1995	0	95	5e-005	1		K.TCNPSDILQMCSFLEGK.-



Immobilized trypsin, digestion 2, measurement 2:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	954
Nominal mass (M$_r$):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 35%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

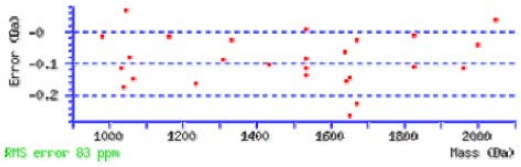
Unformatted sequence string: 686 residues (for pasting into other applications).

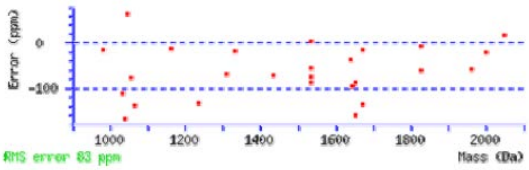
Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
73	9 – 18	618.7000	1235.3854	1235.5492	-0.1638	0	41	17	1		R.WCTISSPEEK.K
40	40 – 47	492.2400	982.4654	982.4794	-0.0139	0	25	3.7e+002	3		K.ATYLDCIK.A
38	100 – 112	479.5500	1435.6282	1435.7307	-0.1025	1	47	3.1	1		K.KGTEFTVNDLQGK.T
76	101 – 112	654.7800	1307.5454	1307.6358	-0.0903	0	72	0.0093	1		K.GTEFTVNDLQGK.T
136	122 – 135	767.8600	1533.7054	1533.8416	-0.1361	0	46	4.3	1		R.SAGWNIPIGTLIHR.G
137	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	37	32	1		R.SAGWNIPIGTLIHR.G
44	122 – 135	512.2500	1533.7282	1533.8416	-0.1134	0	25	4.3e+002	1		R.SAGWNIPIGTLIHR.G
45	122 – 135	512.2600	1533.7582	1533.8416	-0.0834	0	39	17	1		R.SAGWNIPIGTLIHR.G
46	122 – 135	512.2900	1533.8482	1533.8416	0.0066	0	30	1.4e+002	1		R.SAGWNIPIGTLIHR.G
218	136 – 154	980.4300	1958.8454	1958.9585	-0.1131	0	89	0.00017	1		R.GAIEWEGIESGSVEQA
152	155 – 169	821.3700	1640.7254	1640.7869	-0.0614	0	68	0.028	1		K.FFSASCVPGATIEQK.
168	185 – 199	836.2700	1670.5254	1670.7511	-0.2257	0	32	98	1		R.NAPYSGYSGAFHCLK.
169	185 – 199	836.3700	1670.7254	1670.7511	-0.0257	0	71	0.013	1		R.NAPYSGYSGAFHCLK.
49	200 – 209	518.2200	1034.4254	1034.5397	-0.1142	1	27	3.1e+002	1		K.DGKGDVAFVK.H
54	270 – 279	534.1800	1066.3454	1066.4931	-0.1477	0	44	5.5	1		K.AQSDFGVDTK.S
50	333 – 341	521.2000	1040.3854	1040.5614	-0.1760	1	34	53	1		R.KDQLTPSPR.E
82	345 – 355	667.3200	1332.6254	1332.6496	-0.0242	1	25	4.7e+002	1		R.IQWCAVGKDEK.S
56	424 – 438	551.5300	1651.5682	1651.8318	-0.2636	0	56	0.4	1		K.TDERPASYFAVAVAR.
57	424 – 438	551.5700	1651.6882	1651.8318	-0.1436	0	55	0.46	1		K.TDERPASYFAVAVAR.
154	498 – 512	822.3200	1642.6254	1642.7807	-0.1553	0	36	48	1		R.LCQLCQGSGGIPPEK.
51	521 – 529	524.3000	1046.5854	1046.5185	0.0669	0	37	27	1		K.YFGYTGALR.C

207	560 – 574	913.8500	1825.6854	1825.7975	-0.1120	0	61	0.12	1	K.NLQMDDFELLCTDGR.
208	560 – 574	913.9000	1825.7854	1825.7975	-0.0120	0	63	0.074	1	K.NLQMDDFELLCTDGR.
83	583 – 600	683.3700	2047.0882	2047.0520	0.0361	0	35	45	1	R.ECNLAEVPTHAVVVRI
64	625 – 633	581.2500	1160.4854	1160.4994	-0.0140	0	20	1.4e+003	2	K.FMMFESQNK.D
52	661 – 669	529.2600	1056.5054	1056.5855	-0.0801	0	31	1.3e+002	1	K.FYTVISSLK.T
221	670 – 686	1000.4300	1998.8454	1998.8849	-0.0395	0	89	0.00019	1	K.TCNPSDILQMCSFLE(





Immobilized trypsin, digestion 2, measurement 3:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database: NCBInr
Score: 850
Nominal mass (M_r): 77518
Calculated pI: 6.70
Taxonomy: Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file: DATA.TXT
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 34%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

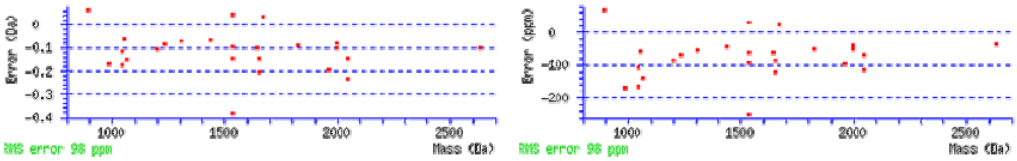
Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
92	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	42	14	1		R.WCTISSPEEK.K
192	48 – 73	878.7400	2633.1982	2633.2973	-0.0991	0	16	4.3e+003	2	U	K.AIANNEADAISLDGGQVFEAGLAPYK.L
109	100 – 112	718.8400	1435.6654	1435.7307	-0.0653	1	21	1.8e+003	1		K.KGTEFTVNDLQGK.T
110	100 – 112	718.8400	1435.6654	1435.7307	-0.0653	1	47	4.4	1		K.KGTEFTVNDLQGK.T
94	101 – 112	654.7900	1307.5654	1307.6358	-0.0703	0	71	0.012	1		K.GTEFTVNDLQGK.T
61	113 – 121	494.6500	987.2854	987.4556	-0.1702	0	40	16	1		K.TSCHTGLGR.S
65	122 – 135	512.1600	1533.4582	1533.8416	-0.3834	0	32	83	1	U	R.SAGWNIPIGTLIHR.G
66	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	36	36	1	U	R.SAGWNIPIGTLIHR.G
149	122 – 135	767.8800	1533.7454	1533.8416	-0.0961	0	31	1.3e+002	2	U	R.SAGWNIPIGTLIHR.G
150	122 – 135	767.9500	1533.8854	1533.8416	0.0439	0	34	68	1	U	R.SAGWNIPIGTLIHR.G
221	136 – 154	980.3900	1958.7654	1958.9585	-0.1931	0	74	0.0059	1		R.GAIEWEGIESGSVEQAVAK.F
167	155 – 169	821.3500	1640.6854	1640.7869	-0.1014	0	43	8.1	1		K.FFSASCVPGATIEQK.L
176	185 – 199	836.4000	1670.7854	1670.7511	0.0343	0	72	0.012	1		R.NAPYSGYSGAFHCLK.D
56	247 – 255	447.3000	892.5854	892.5243	0.0612	0	28	2.1e+002	1		R.VAAHAVVAR.D
74	270 – 279	534.1800	1066.3454	1066.4931	-0.1477	0	43	6.8	1		K.AQSDFGVDTK.S
45	280 – 290	401.1700	1200.4882	1200.5928	-0.1046	0	24	7.8e+002	1		K.SDFHLFGPPGK.K
77	424 – 438	551.5500	1651.6282	1651.8318	-0.2036	0	54	0.59	1		K.TDERPASYFAVAVAR.K
78	424 – 438	551.5700	1651.6882	1651.8318	-0.1436	0	56	0.4	1		K.TDERPASYFAVAVAR.K
69	521 – 529	524.1800	1046.3454	1046.5185	-0.1731	0	28	2.1e+002	1		K.YFGYTGALR.C
70	521 – 529	524.2100	1046.4054	1046.5185	-0.1131	0	37	26	1		K.YFGYTGALR.C

203	560 – 574	913.8600	1825.7054	1825.7975	-0.0920	0	61	0.12	1	K.NLQMDDFELLCTDGR.R
100	583 – 600	683.2800	2046.8182	2047.0520	-0.2339	0	45	5.2	1	R.ECNLAEVPTHAVVVRPEK.A
101	583 – 600	683.3100	2046.9082	2047.0520	-0.1439	0	55	0.54	1	R.ECNLAEVPTHAVVVRPEK.A
71	661 – 669	529.2700	1056.5254	1056.5855	-0.0601	0	35	46	1	K.FYTVISSLK.T
224	670 – 686	1000.4000	1998.7854	1998.8849	-0.0995	0	48	2.4	1	K.TCNPSDILQMCSFLEGK.-
225	670 – 686	1000.4100	1998.8054	1998.8849	-0.0795	0	72	0.01	1	K.TCNPSDILQMCSFLEGK.-



Immobilized trypsin, digestion 3, measurement 1:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database: NCBInr
Score: 715
Nominal mass (M,): 77518
Calculated pI: 6.70
Taxonomy: Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file: DATA.TXT
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 26%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

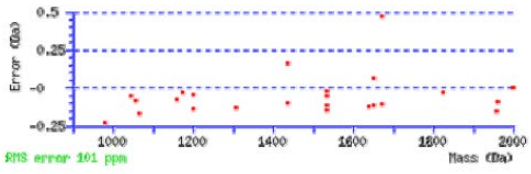
Unformatted sequence string: 686 residues (for pasting into other applications).

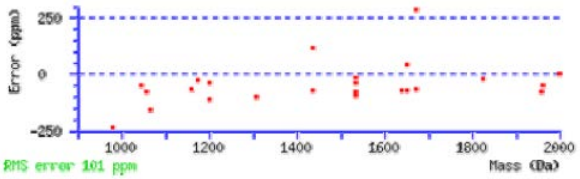
Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
53	40 – 47	492.1300	982.2454	982.4794	-0.2339	0	24	5.2e+002	4		K.ATYLDCIK.A
49	100 – 112	479.5500	1435.6282	1435.7307	-0.1025	1	38	25	1		K.KGTEFTVNDLQGK.T
50	100 – 112	479.6400	1435.8982	1435.7307	0.1675	1	39	19	1		K.KGTEFTVNDLQGK.T
95	101 – 112	654.7600	1307.5054	1307.6358	-0.1303	0	65	0.053	1		K.GTEFTVNDLQGK.T
58	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	25	4e+002	3		R.SAGWNIPIGTLIHR.G
139	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	41	11	1		R.SAGWNIPIGTLIHR.G
140	122 – 135	767.9000	1533.7854	1533.8416	-0.0561	0	49	2.1	1		R.SAGWNIPIGTLIHR.G
59	122 – 135	512.2700	1533.7882	1533.8416	-0.0534	0	30	1.4e+002	1		R.SAGWNIPIGTLIHR.G
60	122 – 135	512.2800	1533.8182	1533.8416	-0.0234	0	38	22	1		R.SAGWNIPIGTLIHR.G
221	136 – 154	980.4100	1958.8054	1958.9585	-0.1531	0	89	0.00017	1		R.GAIEWEGIESGSVEQA
222	136 – 154	980.4400	1958.8654	1958.9585	-0.0931	0	47	2.6	1		R.GAIEWEGIESGSVEQA
158	155 – 169	821.3400	1640.6654	1640.7869	-0.1214	0	51	1.5	1		K.FFSASCVPGATIEQK.
162	185 – 199	836.3300	1670.6454	1670.7511	-0.1057	0	27	3.8e+002	1		R.NAPYSGYSGAFHCLK.
163	185 – 199	836.6200	1671.2254	1670.7511	0.4743	0	26	3.9e+002	1		R.NAPYSGYSGAFHCLK.
68	270 – 279	534.1700	1066.3254	1066.4931	-0.1677	0	46	3.5	1		K.AQSDFGVDTK.S
37	280 – 290	401.1600	1200.4582	1200.5928	-0.1346	0	37	33	1		K.SDFHLFGPPGK.K
90	280 – 290	601.2800	1200.5454	1200.5928	-0.0473	0	33	1e+002	1		K.SDFHLFGPPGK.K
73	424 – 438	551.5800	1651.7182	1651.8318	-0.1136	0	52	0.86	1		K.TDERPASYFAVAVAR.
74	424 – 438	551.6400	1651.8982	1651.8318	0.0664	0	60	0.15	1		K.TDERPASYFAVAVAR.
64	521 – 529	524.2400	1046.4654	1046.5185	-0.0531	0	32	89	1		K.YFGYTGALR.C
195	560 – 574	913.8900	1825.7654	1825.7975	-0.0320	0	64	0.064	1		K.NLQMDDFELLCTDGR.

82	625 – 633	581.2200	1160.4254	1160.4994	-0.0740	0	28	2.5e+002	1	K.FMMFESQNK.D
88	625 – 633	589.2400	1176.4654	1176.4944	-0.0289	0	24	8.7e+002	1	K.FMMFESQNK.D + Oxidation (M)
65	661 – 669	529.2600	1056.5054	1056.5855	-0.0801	0	33	75	1	K.FYTVISSLK.T
230	670 – 686	1000.4500	1998.8854	1998.8849	0.0005	0	74	0.0059	1	K.TCNPSDILQMCSFLE(-





Immobilized trypsin, digestion 3, measurement 2:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	829
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 33%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

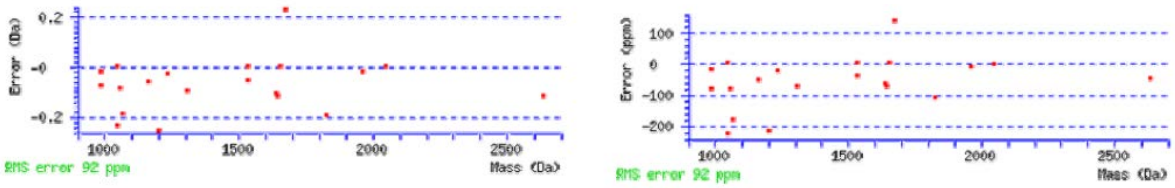
Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
96	9 – 18	618.7700	1235.5254	1235.5492	-0.0238	0	42	14	1		R.WCTISSPEEK.K
59	40 – 47	492.2100	982.4054	982.4794	-0.0739	0	25	4.4e+002	4		K.ATYLDCIK.A
60	40 – 47	492.2400	982.4654	982.4794	-0.0139	0	25	4.5e+002	1		K.ATYLDCIK.A
250	48 – 73	1317.6000	2633.1854	2633.2973	-0.1118	0	118	2.1e-007	1		K.AIANNEADAISLI
105	101 – 112	654.7800	1307.5454	1307.6358	-0.0903	0	74	0.006	1		K.GTEFTVNDLQGK.
64	122 – 135	512.2700	1533.7882	1533.8416	-0.0534	0	25	4.1e+002	4		R.SAGWNIPIGTLI
65	122 – 135	512.2900	1533.8482	1533.8416	0.0066	0	25	4e+002	1		R.SAGWNIPIGTLI
234	136 – 154	980.4800	1958.9454	1958.9585	-0.0131	0	89	0.00017	1		R.GAIEWEGIESGS\
168	155 – 169	821.3500	1640.6854	1640.7869	-0.1014	0	52	1.1	1		K.FFSASCVPGATI
176	185 – 199	836.5000	1670.9854	1670.7511	0.2343	0	24	6.9e+002	1		R.NAPYSGYSGAFH(
75	270 – 279	534.1600	1066.3054	1066.4931	-0.1877	0	38	20	1		K.AQSDFGVDTK.S
45	280 – 290	401.1200	1200.3382	1200.5928	-0.2546	0	27	3.2e+002	6		K.SDFHLFGPPGK.I
79	424 – 438	551.6200	1651.8382	1651.8318	0.0064	0	57	0.31	1		K.TDERPASYFAVA\
80	424 – 438	551.6200	1651.8382	1651.8318	0.0064	0	57	0.27	1		K.TDERPASYFAVA\
169	498 – 512	822.3400	1642.6654	1642.7807	-0.1153	0	50	1.8	1		R.LCQLCQGSGGIPI

70	521 – 529	524.1500	1046.2854	1046.5185	-0.2331	0	30	1.5e+002	1	K.YFGYTGALR.C
71	521 – 529	524.2700	1046.5254	1046.5185	0.0069	0	46	3.6	1	K.YFGYTGALR.C
206	560 – 574	913.8100	1825.6054	1825.7975	-0.1920	0	58	0.23	1	K.NLQMDDFELLCTI
110	583 – 600	683.3600	2047.0582	2047.0520	0.0061	0	36	35	1	R.ECNLAEVPTHAV\
111	583 – 600	683.3600	2047.0582	2047.0520	0.0061	0	38	22	1	R.ECNLAEVPTHAV\
88	625 – 633	581.2300	1160.4454	1160.4994	-0.0540	0	35	50	1	K.FMMFESQNK.D
72	661 – 669	529.2600	1056.5054	1056.5855	-0.0801	0	31	1.3e+002	1	K.FYTVISSLK.T



Immobilized trypsin, digestion 3, measurement 3:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	870
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 33%

Matched peptides shown in ***bold red***.

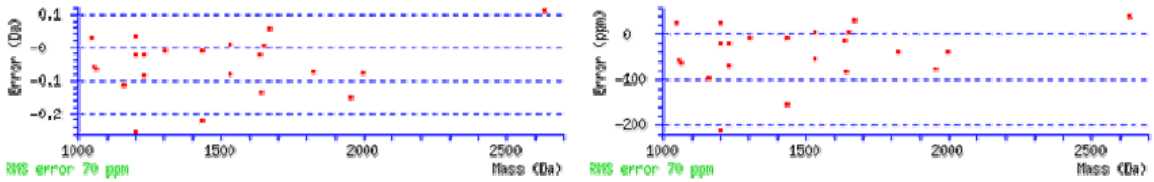
```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHIGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
101	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	42	14	1		R.WCTISSPEEK.K
102	9 – 18	618.7700	1235.5254	1235.5492	-0.0238	0	45	7.9	1		R.WCTISSPEEK.K
197	48 – 73	878.8100	2633.4082	2633.2973	0.1109	0	17	3.7e+003	6		K.AIANNEADAISLDGGQVFEAGLAPYK.L
71	100 – 112	479.5100	1435.5082	1435.7307	-0.2225	1	37	31	1		K.KGTEFTVNDLQGK.T
72	100 – 112	479.5800	1435.7182	1435.7307	-0.0125	1	27	3.2e+002	4		K.KGTEFTVNDLQGK.T
106	101 – 112	654.8200	1307.6254	1307.6358	-0.0103	0	86	0.00036	1		K.GTEFTVNDLQGK.T
78	122 – 135	512.2600	1533.7582	1533.8416	-0.0834	0	40	14	1		R.SAGWNIPIGTLIHR.G
79	122 – 135	512.2900	1533.8482	1533.8416	0.0066	0	32	75	1		R.SAGWNIPIGTLIHR.G
227	136 – 154	980.4100	1958.8054	1958.9585	-0.1531	0	89	0.00017	1		R.GAIEWEGIESGSVEQAVAK.F
171	155 – 169	821.3900	1640.7654	1640.7869	-0.0214	0	55	0.51	1		K.FFSASCVPGATIEQK.L
178	185 – 199	836.4100	1670.8054	1670.7511	0.0543	0	31	1.4e+002	1		R.NAPYSGYSGAFHCLK.D
87	270 – 279	534.2200	1066.4254	1066.4931	-0.0677	0	63	0.069	1		K.AQSDFGVDTK.S
56	280 – 290	401.1200	1200.3382	1200.5928	-0.2546	0	27	4e+002	2		K.SDFHLFGPPGK.K
99	280 – 290	601.3200	1200.6254	1200.5928	0.0327	0	27	4.7e+002	1		K.SDFHLFGPPGK.K
88	424 – 438	551.6200	1651.8382	1651.8318	0.0064	0	63	0.08	1		K.TDERPASYFAVAVAR.K
100	440 – 449	602.2800	1202.5454	1202.5680	-0.0225	0	21	1.9e+003	5		K.DSNVNWNNLK.G
172	498 – 512	822.3300	1642.6454	1642.7807	-0.1353	0	22	1.2e+003	2		R.LCQLCQGSGGIPPEK.C
83	521 – 529	524.2800	1046.5454	1046.5185	0.0269	0	28	2e+002	1		K.YFGYTGALR.C
84	521 – 529	524.2800	1046.5454	1046.5185	0.0269	0	34	51	1		K.YFGYTGALR.C
208	560 – 574	913.8700	1825.7254	1825.7975	-0.0720	0	79	0.0019	1		K.NLQMDDFELLCTDGR.R
94	625 – 633	581.2000	1160.3854	1160.4994	-0.1140	0	29	2e+002	1		K.FMMFESQNK.D
85	661 – 669	529.2700	1056.5254	1056.5855	-0.0601	0	28	2.3e+002	1		K.FYTVISSLK.T
229	670 – 686	1000.4100	1998.8054	1998.8849	-0.0795	0	108	2.6e-006	1		K.TCNPSDILQMCSFLEGK.-



Trypsin/LysC mix, digestion 1, measurement 1:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1520
Nominal mass (M$_r$):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 55%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

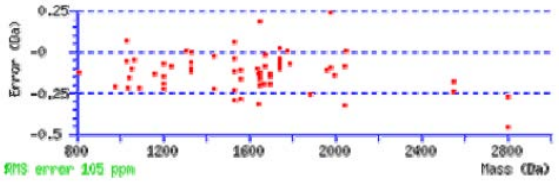
Unformatted sequence string: 686 residues (for pasting into other applications).

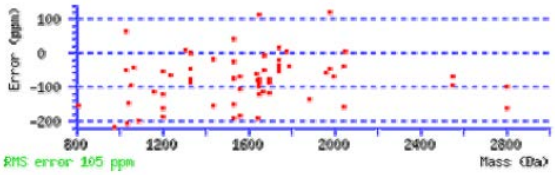
Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
138	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	51	1.7	1		R.WCTISSPEEK.K
74	40 – 47	492.1400	982.2654	982.4794	-0.2139	0	20	1.3e+003	5		K.ATYLDCIK.A
71	100 – 112	479.5100	1435.5082	1435.7307	-0.2225	1	28	2.6e+002	5		K.KGTEFTVNDLQGK.T
171	100 – 112	718.8600	1435.7054	1435.7307	-0.0253	1	60	0.22	1		K.KGTEFTVNDLQGK.T
143	101 – 112	654.8300	1307.6454	1307.6358	0.0097	0	58	0.26	1		K.GTEFTVNDLQGK.T
80	122 – 135	512.1900	1533.5482	1533.8416	-0.2934	0	38	19	1		R.SAGWNIPIGTLIHR.G
81	122 – 135	512.2100	1533.6082	1533.8416	-0.2334	0	32	75	1		R.SAGWNIPIGTLIHR.G
183	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	35	54	1		R.SAGWNIPIGTLIHR.G
82	122 – 135	512.2500	1533.7282	1533.8416	-0.1134	0	49	1.6	1		R.SAGWNIPIGTLIHR.G
184	122 – 135	767.9100	1533.8054	1533.8416	-0.0361	0	48	2.3	1		R.SAGWNIPIGTLIHR.G
185	122 – 135	767.9600	1533.9054	1533.8416	0.0639	0	23	7e+002	1		R.SAGWNIPIGTLIHR.G
240	136 – 154	980.4300	1958.8454	1958.9585	-0.1131	0	79	0.0017	1		R.GAIEWEGIESGSVEQA
202	155 – 169	821.3500	1640.6854	1640.7869	-0.1014	0	40	17	1		K.FFSASCVPGATIEQK.
117	185 – 199	557.8600	1670.5582	1670.7511	-0.1929	0	24	6.3e+002	1		R.NAPYSGYSGAFHCLK.
211	185 – 199	836.3400	1670.6654	1670.7511	-0.0857	0	45	5.3	1		R.NAPYSGYSGAFHCLK.
212	185 – 199	836.3400	1670.6654	1670.7511	-0.0857	0	74	0.0076	1		R.NAPYSGYSGAFHCLK.
118	185 – 199	557.9200	1670.7382	1670.7511	-0.0129	0	23	8.8e+002	1		R.NAPYSGYSGAFHCLK.
90	200 – 209	518.2500	1034.4854	1034.5397	-0.0542	1	44	6.1	1		K.DGKGDVAFVK.H
91	200 – 209	518.3100	1034.6054	1034.5397	0.0658	1	45	4.8	1		K.DGKGDVAFVK.H

235	210 - 233	935.2800	2802.8182	2803.2719	-0.4537	1	36	38	1	K.HTTVNENAPDQKDEYI
236	210 - 233	935.3400	2802.9982	2803.2719	-0.2737	1	40	17	1	K.HTTVNENAPDQKDEYI
50	241 - 246	404.1200	806.2254	806.3493	-0.1239	0	30	1.6e+002	3	K.TCNWAR.V
119	256 - 269	565.8800	1694.6182	1694.8152	-0.1970	1	31	1e+002	1	R.DDNKVEDIWSFLSK.I
213	256 - 269	848.3400	1694.6654	1694.8152	-0.1497	1	61	0.15	1	R.DDNKVEDIWSFLSK.I
214	256 - 269	848.3500	1694.6854	1694.8152	-0.1297	1	57	0.35	1	R.DDNKVEDIWSFLSK.I
100	270 - 279	534.2300	1066.4454	1066.4931	-0.0477	0	48	2.3	1	K.AQSDFGVDTK.S
40	280 - 290	401.1300	1200.3682	1200.5928	-0.2246	0	30	1.8e+002	1	K.SDFHLFGPPGK.K
41	280 - 290	401.1300	1200.3682	1200.5928	-0.2246	0	21	1.4e+003	3	K.SDFHLFGPPGK.K
42	280 - 290	401.1400	1200.3982	1200.5928	-0.1946	0	22	1e+003	6	K.SDFHLFGPPGK.K
135	280 - 290	601.2300	1200.4454	1200.5928	-0.1473	0	24	9.6e+002	1	K.SDFHLFGPPGK.K
136	280 - 290	601.2700	1200.5254	1200.5928	-0.0673	0	30	2.2e+002	1	K.SDFHLFGPPGK.K
92	333 - 341	521.1800	1040.3454	1040.5614	-0.2160	1	28	2e+002	2	R.KDQLTPSPR.E
150	345 - 355	667.2800	1332.5454	1332.6496	-0.1042	1	48	2.7	1	R.IQWCAVGKDEK.S
151	345 - 355	667.3000	1332.5854	1332.6496	-0.0642	1	45	5.6	1	R.IQWCAVGKDEK.S
112	424 - 438	551.5500	1651.6282	1651.8318	-0.2036	0	57	0.29	1	K.TDERPASYFAVAVAR.
205	424 - 438	826.8400	1651.6654	1651.8318	-0.1664	0	22	1e+003	1	K.TDERPASYFAVAVAR.
113	424 - 438	551.5700	1651.6882	1651.8318	-0.1436	0	56	0.36	1	K.TDERPASYFAVAVAR.
206	424 - 438	826.8600	1651.7054	1651.8318	-0.1264	0	30	1.4e+002	1	K.TDERPASYFAVAVAR.
114	424 - 438	551.6800	1652.0182	1651.8318	0.1864	0	53	0.81	1	K.TDERPASYFAVAVAR.
148	439 - 449	666.2800	1330.5454	1330.6629	-0.1175	1	50	1.7	1	R.KDSNVNWNNLK.G
149	439 - 449	666.3400	1330.6654	1330.6629	0.0025	1	64	0.068	1	R.KDSNVNWNNLK.G
93	461 - 474	522.1900	1563.5482	1563.8344	-0.2862	0	36	33	1	R.TAGWVIPMGLIHNR.T
94	461 - 474	522.2300	1563.6682	1563.8344	-0.1662	0	32	79	1	R.TAGWVIPMGLIHNR.T
200	461 - 474	782.8700	1563.7254	1563.8344	-0.1089	0	42	10	1	R.TAGWVIPMGLIHNR.T
215	475 - 497	850.6000	2548.7782	2549.0224	-0.2442	0	30	1.6e+002	1	R.TGTCNFDEYFSEGCAI
250	475 - 497	1275.4300	2548.8454	2549.0224	-0.1769	0	58	0.2	1	R.TGTCNFDEYFSEGCAI
203	498 - 512	822.2400	1642.4654	1642.7807	-0.3153	0	16	3.9e+003	6	R.LCQLCQGSGGIPPEK.
204	498 - 512	822.3300	1642.6454	1642.7807	-0.1353	0	71	0.013	1	R.LCQLCQGSGGIPPEK.
96	521 - 529	524.1900	1046.3654	1046.5185	-0.1531	0	31	1.2e+002	1	K.YFGYTGALR.C
141	535 - 552	630.2200	1887.6382	1887.8963	-0.2581	0	71	0.02	1	K.GDVAFIQHSTVEENTC
144	560 - 575	661.6100	1981.8082	1981.8986	-0.0904	1	27	4.5e+002	1	K.NLQMDDFELLCTDGRI
145	560 - 575	661.6100	1981.8082	1981.8986	-0.0904	1	32	1.5e+002	1	K.NLQMDDFELLCTDGRI
146	560 - 575	661.7200	1982.1382	1981.8986	0.2396	1	19	2.5e+003	5	K.NLQMDDFELLCTDGRI
152	583 - 600	683.2500	2046.7282	2047.0520	-0.3239	0	31	1.3e+002	2	R.ECNLAEVPTHAVVVRI
153	583 - 600	683.3300	2046.9682	2047.0520	-0.0839	0	37	31	1	R.ECNLAEVPTHAVVVRI
154	583 - 600	683.3600	2047.0582	2047.0520	0.0061	0	31	1.2e+002	5	R.ECNLAEVPTHAVVVRI
122	625 - 633	581.1900	1160.3654	1160.4994	-0.1340	0	22	9.1e+002	1	K.FMMFESQNK.D
223	625 - 638	889.4400	1776.8654	1776.8579	0.0076	1	43	8.7	1	K.FMMFESQNKDLLFK.I
133	625 - 638	598.6000	1792.7782	1792.8528	-0.0746	1	26	3.3e+002	3	K.FMMFESQNKDLLFK.I + Oxidation (M)
105	634 - 642	546.7100	1091.4054	1091.6227	-0.2172	1	43	7.9	1	K.DLLFKDLTK.C
217	655 - 669	873.9200	1745.8254	1745.9240	-0.0985	1	68	0.032	1	K.EFLGDKFYTVISSLK.
218	655 - 669	873.9200	1745.8254	1745.9240	-0.0985	1	61	0.16	1	K.EFLGDKFYTVISSLK.
219	655 - 669	873.9300	1745.8454	1745.9240	-0.0785	1	58	0.33	1	K.EFLGDKFYTVISSLK.
129	655 - 669	582.9600	1745.8582	1745.9240	-0.0658	1	23	8.2e+002	2	K.EFLGDKFYTVISSLK.
220	655 - 669	873.9500	1745.8854	1745.9240	-0.0385	1	46	4.6	1	K.EFLGDKFYTVISSLK.
221	655 - 669	873.9500	1745.8854	1745.9240	-0.0385	1	70	0.019	1	K.EFLGDKFYTVISSLK.
130	655 - 669	582.9900	1745.9482	1745.9240	0.0242	1	26	3.6e+002	1	K.EFLGDKFYTVISSLK.
98	661 - 669	529.2500	1056.4854	1056.5855	-0.1001	0	40	14	1	K.FYTVISSLK.T
244	670 - 686	1000.3800	1998.7454	1998.8849	-0.1395	0	81	0.0013	1	K.TCNPSDILQMCSFLEC





Trypsin/LysC mix, digestion 1, measurement 2:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1222
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 49%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

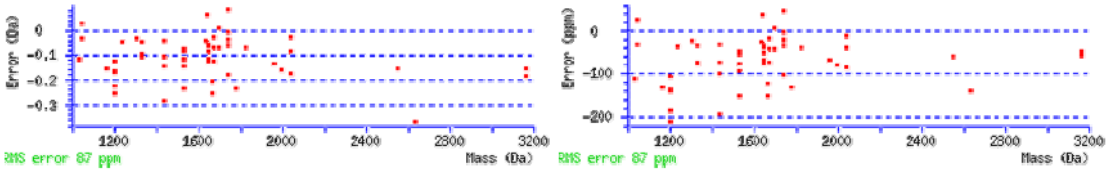
Unformatted sequence string: 686 residues (for pasting into other applications).

Sort peptides by (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
134	9 – 18	618.7600	1235.5054	1235.5492	-0.0438	0	48	3.9	1		R.WCTISSPEEK.K
227	48 – 73	878.6500	2632.9282	2633.2973	-0.3691	0	31	1.3e+002	1		K.AIANNEADAISLDGGQVFEAGLAPYK.L
73	100 – 112	479.4900	1435.4482	1435.7307	-0.2825	1	38	21	1		K.KGTEFTVNDLQGK.T
172	100 – 112	718.8000	1435.5854	1435.7307	-0.1453	1	64	0.084	1		K.KGTEFTVNDLQGK.T
173	100 – 112	718.8200	1435.6254	1435.7307	-0.1053	1	60	0.22	1		K.KGTEFTVNDLQGK.T
74	100 – 112	479.5700	1435.6882	1435.7307	-0.0425	1	40	14	1		K.KGTEFTVNDLQGK.T
138	101 – 112	654.8100	1307.6054	1307.6358	-0.0303	0	75	0.0055	1		K.GTEFTVNDLQGK.T
78	122 – 135	512.2100	1533.6082	1533.8416	-0.2334	0	41	11	1		R.SAGWNIPIGTLIHR.G
79	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	25	4.2e+002	2		R.SAGWNIPIGTLIHR.G
184	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	31	1.3e+002	1		R.SAGWNIPIGTLIHR.G
80	122 – 135	512.2600	1533.7582	1533.8416	-0.0834	0	24	5.8e+002	2		R.SAGWNIPIGTLIHR.G
185	122 – 135	767.8900	1533.7654	1533.8416	-0.0761	0	44	6.6	1		R.SAGWNIPIGTLIHR.G
242	136 – 154	980.4200	1958.8254	1958.9585	-0.1331	0	89	0.00016	1		R.GAIEWEGIESGSVEQAVAK.F
210	155 – 169	821.3800	1640.7454	1640.7869	-0.0414	0	53	0.85	1		K.FFSASCVPGATIEQK.L
107	185 – 199	557.8400	1670.4982	1670.7511	-0.2529	0	37	35	1		R.NAPYSGYSGAFHCLK.D
217	185 – 199	836.2800	1670.5454	1670.7511	-0.2057	0	53	0.92	1		R.NAPYSGYSGAFHCLK.D
218	185 – 199	836.3200	1670.6254	1670.7511	-0.1257	0	58	0.3	1		R.NAPYSGYSGAFHCLK.D
108	185 – 199	557.9000	1670.6782	1670.7511	-0.0729	0	50	2	1		R.NAPYSGYSGAFHCLK.D
219	185 – 199	836.3700	1670.7254	1670.7511	-0.0257	0	57	0.33	1		R.NAPYSGYSGAFHCLK.D
88	200 – 209	518.2200	1034.4254	1034.5397	-0.1142	1	38	26	1		K.DGKGDVAFVK.H
221	256 – 269	848.3800	1694.7454	1694.8152	-0.0697	1	45	5.4	1		R.DDNKVEDIWSFLSK.A
222	256 – 269	848.4200	1694.8254	1694.8152	0.0103	1	52	1	1		R.DDNKVEDIWSFLSK.A
39	280 – 290	401.1200	1200.3382	1200.5928	-0.2546	0	29	2.1e+002	1		K.SDFHLFGPPGK.K
40	280 – 290	401.1300	1200.3682	1200.5928	-0.2246	0	33	88	1		K.SDFHLFGPPGK.K
120	280 – 290	601.2200	1200.4254	1200.5928	-0.1673	0	30	2e+002	1		K.SDFHLFGPPGK.K
41	280 – 290	401.1500	1200.4282	1200.5928	-0.1646	0	37	36	1		K.SDFHLFGPPGK.K
121	280 – 290	601.2400	1200.4654	1200.5928	-0.1273	0	33	1.1e+002	1		K.SDFHLFGPPGK.K
142	345 – 355	667.2800	1332.5454	1332.6496	-0.1042	1	39	22	1		R.IQWCAVGKDEK.S
143	345 – 355	667.3100	1332.6054	1332.6496	-0.0442	1	33	89	2		R.IQWCAVGKDEK.S
245	384 – 414	1054.8100	3161.4082	3161.5923	-0.1841	1	40	14	1		K.IMKGEADAVALDGGLVYTAGVCGLVPVMA
246	384 – 414	1054.8200	3161.4382	3161.5923	-0.1541	1	48	1.9	1		K.IMKGEADAVALDGGLVYTAGVCGLVPVMA
213	424 – 438	826.8600	1651.7054	1651.8318	-0.1264	0	33	71	1		K.TDERPASYFAVAVAR.K
103	424 – 438	551.5800	1651.7182	1651.8318	-0.1136	0	60	0.17	1		K.TDERPASYFAVAVAR.K
104	424 – 438	551.5900	1651.7482	1651.8318	-0.0836	0	53	0.75	1		K.TDERPASYFAVAVAR.K
214	424 – 438	826.8900	1651.7654	1651.8318	-0.0664	0	11	1.1e+004	5		K.TDERPASYFAVAVAR.K

105	424 – 438	551.6000	1651.7782	1651.8318	-0.0536	0	56	0.41	1	K.TDERPASYFAVAVAR.K
106	424 – 438	551.6000	1651.7782	1651.8318	-0.0536	0	62	0.095	1	K.TDERPASYFAVAVAR.K
141	439 – 449	666.2900	1330.5654	1330.6629	-0.0975	1	40	19	1	R.KDSNVNWNNLK.G
223	475 – 497	850.6300	2548.8682	2549.0224	-0.1542	0	37	34	1	R.TGTCNFDEYFSEGCAPGSPPNSR.L
211	498 – 512	822.4300	1642.8454	1642.7807	0.0647	0	73	0.0092	1	R.LCQLCQGSGGIPPEK.C
89	521 – 529	524.2500	1046.4854	1046.5185	-0.0331	0	39	18	1	K.YFGYTGALR.C
90	521 – 529	524.2800	1046.5454	1046.5185	0.0269	0	40	14	1	K.YFGYTGALR.C
239	560 – 574	913.8700	1825.7254	1825.7975	-0.0720	0	73	0.0076	1	K.NLQMDDFELLCTDGR.R
144	583 – 600	683.3000	2046.8782	2047.0520	-0.1739	0	28	2.4e+002	3	R.ECNLAEVPTHAVVVRPEK.A
147	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	29	2e+002	9	R.ECNLAEVPTHAVVVRPEK.A
148	583 – 600	683.3500	2047.0282	2047.0520	-0.0239	0	31	1.2e+002	4	R.ECNLAEVPTHAVVVRPEK.A
111	625 – 633	581.1800	1160.3454	1160.4994	-0.1540	0	39	20	1	K.FMMFESQNK.D
228	625 – 638	889.3200	1776.6254	1776.8579	-0.2324	1	35	55	1	K.FMMFESQNKDLLFK.D
224	655 – 669	873.8800	1745.7454	1745.9240	-0.1785	1	69	0.021	1	K.EFLGDKFYTVISSLK.T
225	655 – 669	873.9400	1745.8654	1745.9240	-0.0585	1	57	0.36	1	K.EFLGDKFYTVISSLK.T
226	655 – 669	873.9500	1745.8854	1745.9240	-0.0385	1	54	0.76	1	K.EFLGDKFYTVISSLK.T
117	655 – 669	582.9700	1745.8882	1745.9240	-0.0358	1	25	5e+002	1	K.EFLGDKFYTVISSLK.T
118	655 – 669	582.9800	1745.9182	1745.9240	-0.0058	1	13	7.7e+003	10	K.EFLGDKFYTVISSLK.T
119	655 – 669	583.0100	1746.0082	1745.9240	0.0842	1	26	3.4e+002	1	K.EFLGDKFYTVISSLK.T
243	670 – 686	1000.3700	1998.7254	1998.8849	-0.1595	0	49	2	1	K.TCNPSDILQMCSFLEGK.-



Trypsin/LysC mix, digestion 1, measurement 3:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1270
Nominal mass (M$_r$):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 51%

Matched peptides shown in ***bold red***.

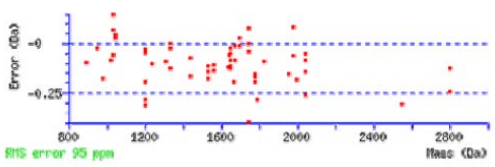
```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

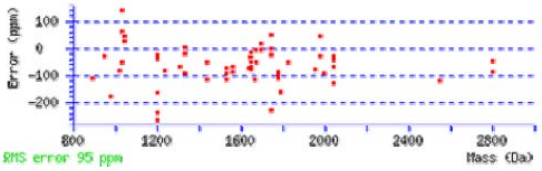
Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
120	9 – 18	618.7300	1235.4454	1235.5492	-0.1038	0	54	0.95	1		R.WCTISSPEEK.K
51	32 – 39	474.7500	947.4854	947.5110	-0.0256	0	48	2.7	1		R.ISLTCVQK.A
54	40 – 47	492.1600	982.3054	982.4794	-0.1739	0	23	6.3e+002	6		K.ATYLDCIK.A
52	100 – 112	479.5300	1435.5682	1435.7307	-0.1625	1	30	1.4e+002	1		K.KGTEFTVNDLQGF
53	100 – 112	479.5600	1435.6582	1435.7307	-0.0725	1	30	1.6e+002	1		K.KGTEFTVNDLQGF
125	101 – 112	654.7800	1307.5454	1307.6358	-0.0903	0	59	0.2	1		K.GTEFTVNDLQGK.
57	122 – 135	512.2300	1533.6682	1533.8416	-0.1734	0	35	44	1		R.SAGWNIPIGTLIF
58	122 – 135	512.2400	1533.6982	1533.8416	-0.1434	0	30	1.3e+002	1		R.SAGWNIPIGTLIF
171	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	24	6.4e+002	4		R.SAGWNIPIGTLIF
241	136 – 154	980.4100	1958.8054	1958.9585	-0.1531	0	83	0.0007	1		R.GAIEWEGIESGSV
200	155 – 169	821.3400	1640.6654	1640.7869	-0.1214	0	40	19	1		K.FFSASCVPGATIF
86	185 – 199	557.8600	1670.5582	1670.7511	-0.1929	0	18	2.5e+003	3		R.NAPYSGYSGAFHC
208	185 – 199	836.3400	1670.6654	1670.7511	-0.0857	0	47	3.6	1		R.NAPYSGYSGAFHC
87	185 – 199	557.9200	1670.7382	1670.7511	-0.0129	0	17	3.4e+003	5		R.NAPYSGYSGAFHC
66	200 – 209	518.2500	1034.4854	1034.5397	-0.0542	1	25	4.3e+002	1		K.DGKGDVAFVK.H
67	200 – 209	518.3100	1034.6054	1034.5397	0.0658	1	39	17	1		K.DGKGDVAFVK.H
68	200 – 209	518.3500	1034.6854	1034.5397	0.1458	1	36	36	1		K.DGKGDVAFVK.H
69	200 – 209	518.3500	1034.6854	1034.5397	0.1458	1	28	2.6e+002	1		K.DGKGDVAFVK.H
234	210 – 233	935.3500	2803.0282	2803.2719	-0.2437	1	28	2.7e+002	1		K.HTTVNENAPDQKI
235	210 – 233	935.3900	2803.1482	2803.2719	-0.1237	1	34	58	1		K.HTTVNENAPDQKI
43	247 – 255	447.2200	892.4254	892.5243	-0.0988	0	33	70	1		R.VAAHAVVAR.D
210	256 – 269	848.4100	1694.8054	1694.8152	-0.0097	1	44	8.1	1		R.DDNKVEDIWSFLS
211	256 – 269	848.4300	1694.8454	1694.8152	0.0303	1	53	0.9	1		R.DDNKVEDIWSFLS
19	280 – 290	401.1000	1200.2782	1200.5928	-0.3146	0	20	1.9e+003	9		K.SDFHLFGPPGK.F
20	280 – 290	401.1100	1200.3082	1200.5928	-0.2846	0	25	6.3e+002	2		K.SDFHLFGPPGK.F
21	280 – 290	401.1400	1200.3982	1200.5928	-0.1946	0	39	23	1		K.SDFHLFGPPGK.F
101	280 – 290	601.2800	1200.5454	1200.5928	-0.0473	0	36	61	1		K.SDFHLFGPPGK.F
102	280 – 290	601.2900	1200.5654	1200.5928	-0.0273	0	40	23	1		K.SDFHLFGPPGK.F
129	345 – 355	667.2700	1332.5254	1332.6496	-0.1242	1	50	1.8	1		R.IQWCAVGKDEK.S
130	345 – 355	667.3200	1332.6254	1332.6496	-0.0242	1	41	14	1		R.IQWCAVGKDEK.S
131	345 – 355	667.3200	1332.6254	1332.6496	-0.0242	1	37	30	1		R.IQWCAVGKDEK.S
203	424 – 438	826.8600	1651.7054	1651.8318	-0.1264	0	20	1.4e+003	1		K.TDERPASYFAVAV
82	424 – 438	551.5800	1651.7182	1651.8318	-0.1136	0	57	0.28	1		K.TDERPASYFAVAV
204	424 – 438	826.8800	1651.7454	1651.8318	-0.0864	0	24	6.1e+002	1		K.TDERPASYFAVAV
83	424 – 438	551.6000	1651.7782	1651.8318	-0.0536	0	60	0.15	1		K.TDERPASYFAVAV
84	424 – 438	551.6100	1651.8082	1651.8318	-0.0236	0	56	0.41	1		K.TDERPASYFAVAV
128	439 – 449	666.3400	1330.6654	1330.6629	0.0025	1	57	0.35	1		R.KDSNVNWNNLK.C
71	461 – 474	522.2400	1563.6982	1563.8344	-0.1362	0	31	1.1e+002	1		R.TAGWVIPMGLIHN
198	461 – 474	782.8700	1563.7254	1563.8344	-0.1089	0	44	6.5	1		R.TAGWVIPMGLIHN
212	475 – 497	850.5800	2548.7182	2549.0224	-0.3042	0	25	5.5e+002	1		R.TGTCNFDEYFSEC
201	498 – 512	822.3700	1642.7254	1642.7807	-0.0553	0	37	31	1		R.LCQLCQGSGGIPE
72	521 – 529	524.2800	1046.5454	1046.5185	0.0269	0	37	25	1		K.YFGYTGALR.C
73	521 – 529	524.2900	1046.5654	1046.5185	0.0469	0	39	18	1		K.YFGYTGALR.C
231	560 – 574	913.8600	1825.7054	1825.7975	-0.0920	0	79	0.0017	1		K.NLQMDDFELLCTI
126	560 – 575	661.6200	1981.8382	1981.8986	-0.0604	1	26	5.7e+002	1		K.NLQMDDFELLCTI
243	560 – 575	992.0000	1981.9854	1981.8986	0.0869	1	16	4.3e+003	5		K.NLQMDDFELLCTI
61	575 – 582	512.7100	1023.4054	1023.4920	-0.0865	1	17	2.3e+003	5		R.RANVMDYR.E
132	583 – 600	683.2700	2046.7882	2047.0520	-0.2639	0	33	85	1		R.ECNLAEVPTHAVV
133	583 – 600	683.3100	2046.9082	2047.0520	-0.1439	0	34	62	1		R.ECNLAEVPTHAVV
134	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	33	85	1		R.ECNLAEVPTHAVV
135	583 – 600	683.3400	2046.9982	2047.0520	-0.0539	0	30	1.5e+002	1		R.ECNLAEVPTHAVV
136	583 – 600	683.3400	2046.9982	2047.0520	-0.0539	0	31	1.1e+002	6		R.ECNLAEVPTHAVV
217	625 – 638	889.3400	1776.6654	1776.8579	-0.1924	1	37	34	1		K.FMMFESQNKDLLI
218	625 – 638	889.3500	1776.6854	1776.8579	-0.1724	1	37	32	2		K.FMMFESQNKDLLI
219	625 – 638	889.3600	1776.7054	1776.8579	-0.1524	1	35	56	2		K.FMMFESQNKDLLI
99	625 – 638	598.5300	1792.5682	1792.8528	-0.2846	1	25	4.6e+002	3		K.FMMFESQNKDLLI + Oxidation (M)
100	625 – 638	598.5300	1792.5682	1792.8528	-0.2846	1	20	1.4e+003	6		K.FMMFESQNKDLLI + Oxidation (M)
97	655 – 669	582.8500	1745.5282	1745.9240	-0.3958	1	20	1.4e+003	2		K.EFLGDKFYTVISS
213	655 – 669	873.9500	1745.8854	1745.9240	-0.0385	1	50	2	1		K.EFLGDKFYTVISS
214	655 – 669	873.9700	1745.9254	1745.9240	0.0015	1	45	5.7	1		K.EFLGDKFYTVISS
215	655 – 669	874.0100	1746.0054	1745.9240	0.0815	1	69	0.023	1		K.EFLGDKFYTVISS
216	655 – 669	874.0100	1746.0054	1745.9240	0.0815	1	65	0.059	1		K.EFLGDKFYTVISS
244	670 – 686	1000.3600	1998.7054	1998.8849	-0.1795	0	80	0.0015	1		K.TCNPSDILQMCSI –





Trypsin/LysC mix, digestion 2, measurement 1:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	602
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 24%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
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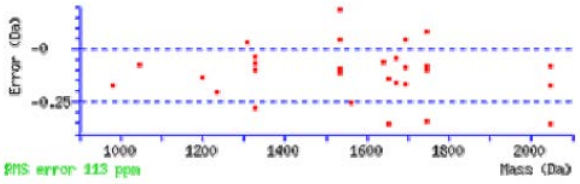
Unformatted sequence string: 686 residues (for pasting into other applications).

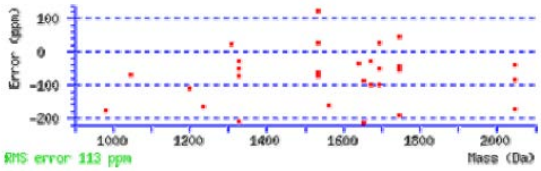
Sort peptides by (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
151	9 – 18	618.6800	1235.3454	1235.5492	-0.2038	0	37	47	1		R.WCTISSPEEK.K
104	40 – 47	492.1600	982.3054	982.4794	-0.1739	0	24	4.8e+002	2		K.ATYLDCIK.A
153	101 – 112	654.8400	1307.6654	1307.6358	0.0297	0	74	0.0056	1		K.GTEFTVNDLQGK.T
106	122 – 135	512.2500	1533.7282	1533.8416	-0.1134	0	35	41	1		R.SAGWNIPIGTLIHR
174	122 – 135	767.8800	1533.7454	1533.8416	-0.0961	0	26	3.7e+002	4		R.SAGWNIPIGTLIHR
177	122 – 135	767.9500	1533.8854	1533.8416	0.0439	0	32	90	2		R.SAGWNIPIGTLIHR
107	122 – 135	512.3500	1534.0282	1533.8416	0.1866	0	38	22	1		R.SAGWNIPIGTLIHR
220	155 – 169	821.3700	1640.7254	1640.7869	-0.0614	0	52	1	1		K.FFSASCVPGATIEQ
122	185 – 199	557.8700	1670.5882	1670.7511	-0.1629	0	24	6.7e+002	2		R.NAPYSGYSGAFHCL
227	185 – 199	836.3600	1670.7054	1670.7511	-0.0457	0	50	1.9	1		R.NAPYSGYSGAFHCL

228	256 – 269	848.3300	1694.6454	1694.8152	-0.1697	1	45	5.4	1	R.DDNKVEDIWSFLSK
229	256 – 269	848.3700	1694.7254	1694.8152	-0.0897	1	41	13	1	R.DDNKVEDIWSFLSK
123	256 – 269	565.9600	1694.8582	1694.8152	0.0430	1	36	33	1	R.DDNKVEDIWSFLSK
78	280 – 290	401.1600	1200.4582	1200.5928	-0.1346	0	21	1.4e+003	4	K.SDFHLFGPPGK.K
93	280 – 291	443.8100	1328.4082	1328.6877	-0.2796	1	27	3.1e+002	1	K.SDFHLFGPPGKK.D
94	280 – 291	443.8100	1328.4082	1328.6877	-0.2796	1	23	7.7e+002	1	K.SDFHLFGPPGKK.D
95	280 – 291	443.8700	1328.5882	1328.6877	-0.0996	1	39	21	1	K.SDFHLFGPPGKK.D
96	280 – 291	443.8800	1328.6182	1328.6877	-0.0696	1	29	1.9e+002	1	K.SDFHLFGPPGKK.D
97	280 – 291	443.8900	1328.6482	1328.6877	-0.0396	1	24	6e+002	1	K.SDFHLFGPPGKK.D
118	424 – 438	551.5000	1651.4782	1651.8318	-0.3536	0	49	2.1	1	K.TDERPASYFAVAVA
119	424 – 438	551.5700	1651.6882	1651.8318	-0.1436	0	54	0.63	1	K.TDERPASYFAVAVA
120	424 – 438	551.5700	1651.6882	1651.8318	-0.1436	0	62	0.096	1	K.TDERPASYFAVAVA
111	461 – 474	522.2000	1563.5782	1563.8344	-0.2562	0	19	1.7e+003	7	R.TAGWVIPMGLIHNR
156	583 – 600	683.2400	2046.6982	2047.0520	-0.3539	0	26	3.7e+002	7	R.ECNLAEVPTHAVVV
158	583 – 600	683.3000	2046.8782	2047.0520	-0.1739	0	30	1.5e+002	1	R.ECNLAEVPTHAVVV
159	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	25	4.8e+002	3	R.ECNLAEVPTHAVVV
230	655 – 669	873.8000	1745.5854	1745.9240	-0.3385	1	41	15	1	K.EFLGDKFYTVISSL
231	655 – 669	873.9200	1745.8254	1745.9240	-0.0985	1	72	0.013	1	K.EFLGDKFYTVISSL
232	655 – 669	873.9300	1745.8454	1745.9240	-0.0785	1	70	0.021	1	K.EFLGDKFYTVISSL
233	655 – 669	874.0100	1746.0054	1745.9240	0.0815	1	27	3.6e+002	1	K.EFLGDKFYTVISSL





Trypsin/LysC mix, digestion 2, measurement 2:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1037
Nominal mass (M$_r$):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 48%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
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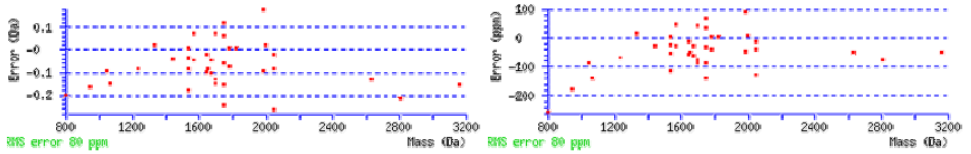
Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
144	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	54	0.89	1		R.WCTISSPEEK.K
85	32 – 39	474.6800	947.3454	947.5110	-0.1656	0	40	14	1		R.ISLTCVQK.A
217	48 – 73	878.7300	2633.1682	2633.2973	-0.1291	0	32	1.1e+002	1		K.AIANNEADAISLDGGQVFEAGLAPYK.L
86	100 – 112	479.5700	1435.6882	1435.7307	-0.0425	1	35	46	2		K.KGTEFTVNDLQGK.T
165	122 – 135	767.8400	1533.6654	1533.8416	-0.1761	0	27	2.9e+002	5		R.SAGWNIPIGTLIHR.G
88	122 – 135	512.2600	1533.7582	1533.8416	-0.0834	0	32	92	1		R.SAGWNIPIGTLIHR.G
166	122 – 135	767.9100	1533.8054	1533.8416	-0.0361	0	38	24	2		R.SAGWNIPIGTLIHR.G
89	122 – 135	512.2900	1533.8482	1533.8416	0.0066	0	41	10	1		R.SAGWNIPIGTLIHR.G
205	155 – 169	821.3900	1640.7654	1640.7869	-0.0214	0	64	0.064	1		K.FFSASCVPGATIEQK.L
108	185 – 199	557.8900	1670.6482	1670.7511	-0.1029	0	46	4.6	1		R.NAPYSGYSGAFHCLK.D
210	185 – 199	836.3600	1670.7054	1670.7511	-0.0457	0	52	1.1	1		R.NAPYSGYSGAFHCLK.D
238	210 – 233	935.3600	2803.0582	2803.2719	-0.2137	1	26	3.9e+002	1		K.HTTVNENAPDQKDEYELLCLDGSR.Q
72	241 – 246	404.0800	806.1454	806.3493	-0.2039	0	27	2.9e+002	5		K.TCNWAR.V
212	256 – 269	848.3400	1694.6654	1694.8152	-0.1497	1	44	6.9	1		R.DDNKVEDIWSFLSK.A
213	256 – 269	848.3500	1694.6854	1694.8152	-0.1297	1	50	1.7	1		R.DDNKVEDIWSFLSK.A
109	256 – 269	565.9700	1694.8882	1694.8152	0.0730	1	33	68	1		R.DDNKVEDIWSFLSK.A
110	256 – 269	565.9700	1694.8882	1694.8152	0.0730	1	39	17	1		R.DDNKVEDIWSFLSK.A
97	270 – 279	534.1800	1066.3454	1066.4931	-0.1477	0	55	0.41	1		K.AQSDFGVDTK.S
81	280 – 291	443.9100	1328.7082	1328.6877	0.0204	1	34	59	1		K.SDFHLFGPPGKK.D
244	384 – 414	1054.8200	3161.4382	3161.5923	-0.1541	1	36	33	1		K.IMKGEADAVALDGGLVYTAGVCGLVPVMA
104	424 – 438	551.5900	1651.7482	1651.8318	-0.0836	0	57	0.33	1		K.TDERPASYFAVAVAR.K
95	461 – 474	522.2700	1563.7882	1563.8344	-0.0462	0	25	4.3e+002	3		R.TAGWVIPMGLIHNR.T
198	461 – 474	782.9600	1563.9054	1563.8344	0.0711	0	21	1.3e+003	5		R.TAGWVIPMGLIHNR.T
206	498 – 512	822.3500	1642.6854	1642.7807	-0.0953	0	31	1.3e+002	1		R.LCQLCQGSGGIPPEK.C
96	521 – 529	524.2200	1046.4254	1046.5185	-0.0931	0	39	17	1		K.YFGYTGALR.C
221	560 – 574	913.9100	1825.8054	1825.7975	0.0080	0	50	1.3	1		K.NLQMDDFELLCTDGR.R
146	560 – 575	661.6100	1981.8082	1981.8986	-0.0904	1	34	89	1		K.NLQMDDFELLCTDGRR.A
147	560 – 575	661.7000	1982.0782	1981.8986	0.1796	1	24	8.6e+002	1		K.NLQMDDFELLCTDGRR.A
148	583 – 600	683.2700	2046.7882	2047.0520	-0.2639	0	29	2.1e+002	8		R.ECNLAEVPTHAVVVRPEK.A
149	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	35	44	2		R.ECNLAEVPTHAVVVRPEK.A
150	583 – 600	683.3500	2047.0282	2047.0520	-0.0239	0	27	3e+002	10		R.ECNLAEVPTHAVVVRPEK.A
219	625 – 638	889.4000	1776.7854	1776.8579	-0.0724	1	26	4e+002	2		K.FMMFESQNKDLLFK.D
220	625 – 638	889.4400	1776.8654	1776.8579	0.0076	1	39	23	1		K.FMMFESQNKDLLFK.D
120	655 – 669	582.9000	1745.6782	1745.9240	-0.2458	1	21	1.1e+003	1		K.EFLGDKFYTVISSLK.T
122	655 – 669	582.9300	1745.7682	1745.9240	-0.1558	1	18	2.1e+003	3		K.EFLGDKFYTVISSLK.T
214	655 – 669	873.9400	1745.8654	1745.9240	-0.0585	1	57	0.35	1		K.EFLGDKFYTVISSLK.T
215	655 – 669	874.0000	1745.9854	1745.9240	0.0615	1	70	0.021	1		K.EFLGDKFYTVISSLK.T
216	655 – 669	874.0300	1746.0454	1745.9240	0.1215	1	64	0.08	1		K.EFLGDKFYTVISSLK.T
243	670 – 686	1000.4600	1998.9054	1998.8849	0.0205	0	108	2.3e-006	1		K.TCNPSDILQMCSFLEGK.-



Trypsin/LysC mix, digestion 2, measurement 3:

MATRIX SCIENCE **MASCOT Search Results**

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1000
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 37%

Matched peptides shown in ***bold red***.

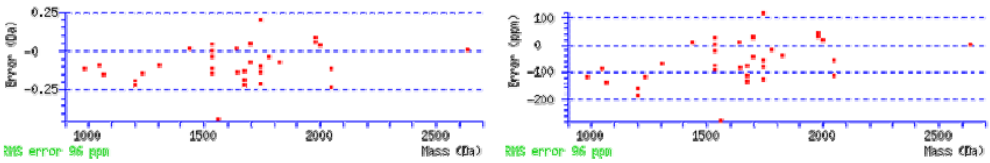
```
   1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
  51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
 101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
 151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
 201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
 251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
 301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
 351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
 401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
 451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
 501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
 551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
 601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
 651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
130	9 - 18	618.7100	1235.4054	1235.5492	-0.1438	0	43	11	1		R.WCTISSPEEK.K
75	40 - 47	492.1900	982.3654	982.4794	-0.1139	0	28	2.1e+002	3		K.ATYLDCIK.A
250	48 - 73	1317.6600	2633.3054	2633.2973	0.0082	0	83	0.00057	1		K.AIANNEADAISLDGGQVFEAGLAPYK.L
74	100 - 112	479.5900	1435.7482	1435.7307	0.0175	1	38	24	1		K.KGTEFTVNDLQGK.T
132	101 - 112	654.7800	1307.5454	1307.6358	-0.0903	0	75	0.0054	1		K.GTEFTVNDLQGK.T
76	122 - 135	512.2400	1533.6982	1533.8416	-0.1434	0	39	18	1		R.SAGWNIPIGTLIHR.G
154	122 - 135	767.8700	1533.7254	1533.8416	-0.1161	0	45	5.1	1		R.SAGWNIPIGTLIHR.G
77	122 - 135	512.2500	1533.7282	1533.8416	-0.1134	0	29	1.6e+002	1		R.SAGWNIPIGTLIHR.G
155	122 - 135	767.9100	1533.8054	1533.8416	-0.0361	0	39	19	1		R.SAGWNIPIGTLIHR.G
156	122 - 135	767.9300	1533.8454	1533.8416	0.0039	0	27	3e+002	2		R.SAGWNIPIGTLIHR.G
157	122 - 135	767.9500	1533.8854	1533.8416	0.0439	0	30	1.5e+002	5		R.SAGWNIPIGTLIHR.G
196	155 - 169	821.4100	1640.8054	1640.7869	0.0186	0	56	0.48	1		K.FFSASCVPGATIEQK.L
96	185 - 199	557.8500	1670.5282	1670.7511	-0.2229	0	39	21	1		R.NAPYSGYSGAFHCLK.D
204	185 - 199	836.2900	1670.5654	1670.7511	-0.1857	0	50	1.8	1		R.NAPYSGYSGAFHCLK.D
205	185 - 199	836.3200	1670.6254	1670.7511	-0.1257	0	58	0.31	1		R.NAPYSGYSGAFHCLK.D
207	256 - 269	848.3800	1694.7454	1694.8152	-0.0697	1	45	5.5	1		R.DDNKVEDIWSFLSK.A
97	256 - 269	565.9600	1694.8582	1694.8152	0.0430	1	41	11	1		R.DDNKVEDIWSFLSK.A
208	256 - 269	848.4400	1694.8654	1694.8152	0.0503	1	52	1	1		R.DDNKVEDIWSFLSK.A
87	270 - 279	534.1800	1066.3454	1066.4931	-0.1477	0	49	1.9	1		K.AQSDFGVDTK.S
57	280 - 290	401.1300	1200.3682	1200.5928	-0.2246	0	30	2e+002	1		K.SDFHLFGPPGK.K
58	280 - 290	401.1400	1200.3982	1200.5928	-0.1946	0	26	4.7e+002	1		K.SDFHLFGPPGK.K
85	461 - 474	522.1400	1563.3982	1563.8344	-0.4362	0	34	60	2		R.TAGWVIPMGLIHNR.T
197	498 - 512	822.3300	1642.6454	1642.7807	-0.1353	0	47	3.2	1		R.LCQLCQGSGGIPPEK.C
86	521 - 529	524.2200	1046.4254	1046.5185	-0.0931	0	37	28	1		K.YFGYTGALR.C
216	560 - 574	913.8700	1825.7254	1825.7975	-0.0720	0	68	0.022	1		K.NLQMDDFELLCTDGR.R
134	560 - 575	661.6600	1981.9582	1981.8986	0.0596	1	22	1.5e+003	1		K.NLQMDDFELLCTDGRR.A
135	560 - 575	661.6700	1981.9882	1981.8986	0.0896	1	30	2e+002	1		K.NLQMDDFELLCTDGRR.A
136	583 - 600	683.2800	2046.8182	2047.0520	-0.2339	0	28	2.3e+002	6		R.ECNLAEVPTHAVVVRPEK.A
137	583 - 600	683.2800	2046.8182	2047.0520	-0.2339	0	23	7.6e+002	8		R.ECNLAEVPTHAVVVRPEK.A
139	583 - 600	683.3200	2046.9382	2047.0520	-0.1139	0	35	45	1		R.ECNLAEVPTHAVVVRPEK.A
140	583 - 600	683.3200	2046.9382	2047.0520	-0.1139	0	35	45	1		R.ECNLAEVPTHAVVVRPEK.A
213	625 - 638	889.4200	1776.8254	1776.8579	-0.0324	1	28	2.8e+002	2		K.FMMFESQNKDLLFK.D
104	655 - 669	582.9100	1745.7082	1745.9240	-0.2158	1	15	4.1e+003	5		K.EFLGDKFYTVISSLK.T
210	655 - 669	873.9000	1745.7854	1745.9240	-0.1385	1	70	0.019	1		K.EFLGDKFYTVISSLK.T
211	655 - 669	873.9200	1745.8254	1745.9240	-0.0985	1	54	0.73	1		K.EFLGDKFYTVISSLK.T
105	655 - 669	583.0500	1746.1282	1745.9240	0.2042	1	17	3e+003	6		K.EFLGDKFYTVISSLK.T
244	670 - 686	1000.4700	1998.9254	1998.8849	0.0405	0	95	4.8e-005	1		K.TCNPSDILQMCSFLEGK.-



Trypsin/LysC mix, digestion 3, measurement 1:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1435
Nominal mass (M_r):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 53%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

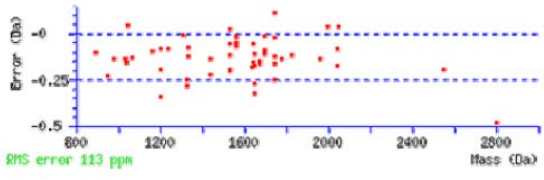
Unformatted sequence string: **686 residues** (for pasting into other applications).

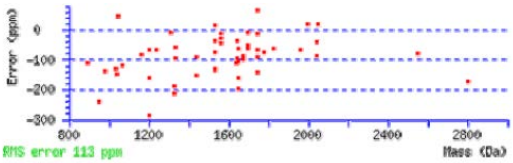
Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
114	9 – 18	618.7400	1235.4654	1235.5492	-0.0838	0	41	19	1		R.WCTISSPEEK.K
56	32 – 39	474.6500	947.2854	947.5110	-0.2256	0	48	2.5	1		R.ISLTCVQK.A
60	40 – 47	492.1800	982.3454	982.4794	-0.1339	0	25	4e+002	4		K.ATYLDCIK.A
57	100 – 112	479.5100	1435.5082	1435.7307	-0.2225	1	46	3.7	1		K.KGTEFTVNDLQGK
58	100 – 112	479.5400	1435.5982	1435.7307	-0.1325	1	42	11	2		K.KGTEFTVNDLQGK
117	101 – 112	654.8200	1307.6254	1307.6358	-0.0103	0	61	0.13	1		K.GTEFTVNDLQGK.
61	122 – 135	512.2200	1533.6382	1533.8416	-0.2034	0	35	40	1		R.SAGWNIPIGTLIH
148	122 – 135	767.8300	1533.6454	1533.8416	-0.1961	0	45	5.5	1		R.SAGWNIPIGTLIH
149	122 – 135	767.8700	1533.7254	1533.8416	-0.1161	0	38	24	2		R.SAGWNIPIGTLIH
62	122 – 135	512.2700	1533.7882	1533.8416	-0.0534	0	35	43	1		R.SAGWNIPIGTLIH
150	122 – 135	767.9400	1533.8654	1533.8416	0.0239	0	37	29	2		R.SAGWNIPIGTLIH
239	136 – 154	980.4200	1958.8254	1958.9585	-0.1331	0	55	0.44	1		R.GAIEWEGIESGSV
184	155 – 169	821.3100	1640.6054	1640.7869	-0.1814	0	53	0.95	1		K.FFSASCVPGATIH
82	185 – 199	557.8700	1670.5882	1670.7511	-0.1629	0	44	7.7	1		R.NAPYSGYSGAFHC
192	185 – 199	836.3100	1670.6054	1670.7511	-0.1457	0	58	0.3	1		R.NAPYSGYSGAFHC
67	200 – 209	518.2100	1034.4054	1034.5397	-0.1342	1	45	5.1	1		K.DGKGDVAFVK.H
234	210 – 233	935.2700	2802.7882	2803.2719	-0.4837	1	50	1.6	1		K.HTTVNENAPDQKD
52	247 – 255	447.2200	892.4254	892.5243	-0.0988	0	34	51	1		R.VAAHAVVAR.D
195	256 – 269	848.3600	1694.7054	1694.8152	-0.1097	1	44	7.5	1		R.DDNKVEDIWSFLS
196	256 – 269	848.3700	1694.7254	1694.8152	-0.0897	1	49	2.3	1		R.DDNKVEDIWSFLS
83	256 – 269	565.9400	1694.7982	1694.8152	-0.0170	1	40	14	1		R.DDNKVEDIWSFLS
74	270 – 279	534.1900	1066.3654	1066.4931	-0.1277	0	49	1.7	1		K.AQSDFGVDTK.S
34	280 – 290	401.0900	1200.2482	1200.5928	-0.3446	0	28	2.7e+002	1		K.SDFHLFGPPGK.F

36	280 – 290	401.1400	1200.3982	1200.5928	-0.1946	0	23	8.7e+002	3	K.SDFHLFGPPGK.I
47	280 – 291	443.8100	1328.4082	1328.6877	-0.2796	1	38	23	1	K.SDFHLFGPPGKK.
48	280 – 291	443.8200	1328.4382	1328.6877	-0.2496	1	34	61	1	K.SDFHLFGPPGKK.
49	280 – 291	443.8200	1328.4382	1328.6877	-0.2496	1	40	15	1	K.SDFHLFGPPGKK.
68	333 – 341	521.2100	1040.4054	1040.5614	-0.1560	1	40	14	2	R.KDQLTPSPR.E
120	345 – 355	667.2700	1332.5254	1332.6496	-0.1242	1	37	32	1	R.IQWCAVGKDEK.S
78	424 – 438	551.5100	1651.5082	1651.8318	-0.3236	0	56	0.4	1	K.TDERPASYFAVA\
188	424 – 438	826.7900	1651.5654	1651.8318	-0.2664	0	16	4e+003	2	K.TDERPASYFAVA\
79	424 – 438	551.5600	1651.6582	1651.8318	-0.1736	0	48	2.5	1	K.TDERPASYFAVA\
189	424 – 438	826.8700	1651.7254	1651.8318	-0.1064	0	28	2.5e+002	1	K.TDERPASYFAVA\
119	439 – 449	666.3000	1330.5854	1330.6629	-0.0775	1	55	0.56	1	R.KDSNVNWNNLK.G
91	440 – 449	602.2500	1202.4854	1202.5680	-0.0825	0	38	41	2	K.DSNVNWNNLK.G
175	461 – 474	782.8900	1563.7654	1563.8344	-0.0689	0	37	26	1	R.TAGWVIPMGLIHN
70	461 – 474	522.2700	1563.7882	1563.8344	-0.0462	0	37	26	1	R.TAGWVIPMGLIHN
71	461 – 474	522.2800	1563.8182	1563.8344	-0.0162	0	36	33	1	R.TAGWVIPMGLIHN
250	475 – 497	1275.4200	2548.8254	2549.0224	-0.1969	0	70	0.013	1	R.TGTCNFDEYFSEC
186	498 – 512	822.3200	1642.6254	1642.7807	-0.1553	0	50	1.6	1	R.LCQLCQGSGGIPI
187	498 – 512	822.3700	1642.7254	1642.7807	-0.0553	0	36	40	1	R.LCQLCQGSGGIPI
72	521 – 529	524.2900	1046.5654	1046.5185	0.0469	0	33	70	1	K.YFGYTGALR.C
219	560 – 574	913.8500	1825.6854	1825.7975	-0.1120	0	70	0.016	1	K.NLQMDDFELLCTI
122	583 – 600	683.3000	2046.8782	2047.0520	-0.1739	0	33	80	1	R.ECNLAEVPTHAV\
123	583 – 600	683.3300	2046.9682	2047.0520	-0.0839	0	31	1.2e+002	9	R.ECNLAEVPTHAV\
124	583 – 600	683.3700	2047.0882	2047.0520	0.0361	0	24	6.4e+002	3	R.ECNLAEVPTHAV\
84	625 – 633	581.2100	1160.4054	1160.4994	-0.0940	0	22	9.2e+002	1	K.FMMFESQNK.D
205	625 – 638	889.3700	1776.7254	1776.8579	-0.1324	1	44	6.4	1	K.FMMFESQNKDLLI
88	655 – 669	582.9000	1745.6782	1745.9240	-0.2458	1	22	1e+003	1	K.EFLGDKFYTVISS
89	655 – 669	582.9000	1745.6782	1745.9240	-0.2458	1	22	1e+003	1	K.EFLGDKFYTVISS
199	655 – 669	873.8900	1745.7654	1745.9240	-0.1585	1	53	0.82	1	K.EFLGDKFYTVISS
200	655 – 669	873.9100	1745.8054	1745.9240	-0.1185	1	65	0.053	1	K.EFLGDKFYTVISS
201	655 – 669	873.9600	1745.9054	1745.9240	-0.0185	1	31	1.6e+002	1	K.EFLGDKFYTVISS
90	655 – 669	583.0200	1746.0382	1745.9240	0.1142	1	27	3.2e+002	1	K.EFLGDKFYTVISS
241	670 – 686	1000.4700	1998.9254	1998.8849	0.0405	0	61	0.13	1	K.TCNPSDILQMCSI





Trypsin/LysC mix, digestion 3, measurement 2:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

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Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 60%

Matched peptides shown in ***bold red***.

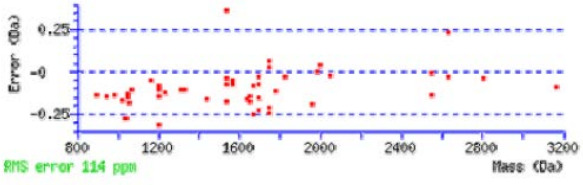
```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

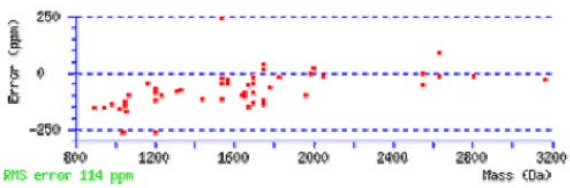
Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start - End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
120	9 - 18	618.7200	1235.4254	1235.5492	-0.1238	0	60	0.23	1		R.WCTISSPEEK.K
62	32 - 39	474.6900	947.3654	947.5110	-0.1456	0	31	1.1e+002	3		R.ISLTCVQK.A
65	40 - 47	492.1800	982.3454	982.4794	-0.1339	0	25	4.4e+002	3		K.ATYLDCIK.A
250	48 - 73	1317.6400	2633.2654	2633.2973	-0.0318	0	44	4.2	1		K.AIANNEADAISLI
204	48 - 73	878.8500	2633.5282	2633.2973	0.2309	0	32	98	1		K.AIANNEADAISLI
63	100 - 112	479.5300	1435.5682	1435.7307	-0.1625	1	48	2.2	1		K.KGTEFTVNDLQGK
123	101 - 112	654.7700	1307.5254	1307.6358	-0.1103	0	74	0.0059	1		K.GTEFTVNDLQGK.
66	122 - 135	512.2300	1533.6682	1533.8416	-0.1734	0	29	1.7e+002	1		R.SAGWNIPIGTLIH
155	122 - 135	767.8900	1533.7654	1533.8416	-0.0761	0	26	3.9e+002	4		R.SAGWNIPIGTLIH
156	122 - 135	767.9100	1533.8054	1533.8416	-0.0361	0	37	29	2		R.SAGWNIPIGTLIH
157	122 - 135	767.9100	1533.8054	1533.8416	-0.0361	0	40	17	2		R.SAGWNIPIGTLIH
67	122 - 135	512.4100	1534.2082	1533.8416	0.3666	0	40	12	1		R.SAGWNIPIGTLIH
234	136 - 154	980.3900	1958.7654	1958.9585	-0.1931	0	77	0.003	1		R.GAIEWEGIESGSV
190	155 - 169	821.3200	1640.6254	1640.7869	-0.1614	0	54	0.68	1		K.FFSASCVPGATIH
85	185 - 199	557.8400	1670.4982	1670.7511	-0.2529	0	18	3.1e+003	2		R.NAPYSGYSGAFHC
197	185 - 199	836.3400	1670.6654	1670.7511	-0.0857	0	65	0.05	1		R.NAPYSGYSGAFHC
73	200 - 209	518.1400	1034.2654	1034.5397	-0.2742	1	43	7.2	1		K.DGKGDVAFVK.H
230	210 - 233	935.4200	2803.2382	2803.2719	-0.0337	1	40	16	1		K.HTTVNENAPDQKI
57	247 - 255	447.2000	892.3854	892.5243	-0.1388	0	33	70	1		R.VAAHAVVAR.D
86	256 - 269	565.8700	1694.5882	1694.8152	-0.2270	1	28	2.4e+002	1		R.DDNKVEDIWSFLS
198	256 - 269	848.3400	1694.6654	1694.8152	-0.1497	1	56	0.44	1		R.DDNKVEDIWSFLS
87	256 - 269	565.9200	1694.7382	1694.8152	-0.0770	1	37	30	1		R.DDNKVEDIWSFLS
79	270 - 279	534.2000	1066.3854	1066.4931	-0.1077	0	48	2	1		K.AQSDFGVDTK.S
46	280 - 290	401.1000	1200.2782	1200.5928	-0.3146	0	31	1.4e+002	1		K.SDFHLFGPPGK.K
96	280 - 290	601.2300	1200.4454	1200.5928	-0.1473	0	27	4.8e+002	1		K.SDFHLFGPPGK.K
47	280 - 290	401.1700	1200.4882	1200.5928	-0.1046	0	25	5.7e+002	1		K.SDFHLFGPPGK.K
74	333 - 341	521.1500	1040.2854	1040.5614	-0.2760	1	39	17	4		R.KDQLTPSPR.E
125	345 - 355	667.2800	1332.5454	1332.6496	-0.1042	1	40	18	1		R.IQWCAVGKDEK.S
237	384 - 414	1054.8400	3161.4982	3161.5923	-0.0941	1	21	1.1e+003	1		K.IMKGEADAVALDG
81	424 - 438	551.5600	1651.6582	1651.8318	-0.1736	0	57	0.33	1		K.TDERPASYFAVAV
82	424 - 438	551.5700	1651.6882	1651.8318	-0.1436	0	48	2.7	1		K.TDERPASYFAVAV
97	440 - 449	602.2500	1202.4854	1202.5680	-0.0825	0	32	1.5e+002	2		K.DSNVNWNNLK.G
75	461 - 474	522.2600	1563.7582	1563.8344	-0.0762	0	33	74	1		R.TAGWVIPMGLIHN
182	461 - 474	782.9000	1563.7854	1563.8344	-0.0489	0	48	2.4	1		R.TAGWVIPMGLIHN
249	475 - 497	1275.4500	2548.8854	2549.0224	-0.1369	0	73	0.0067	1		R.TGTCNFDEYFSEC
200	475 - 497	850.6800	2549.0182	2549.0224	-0.0042	0	28	2.6e+002	1		R.TGTCNFDEYFSEC
76	521 - 529	524.1900	1046.3654	1046.5185	-0.1531	0	39	18	1		K.YFGYTGALR.C
77	521 - 529	524.2000	1046.3854	1046.5185	-0.1331	0	40	13	1		K.YFGYTGALR.C
215	560 - 574	913.8900	1825.7654	1825.7975	-0.0320	0	68	0.026	1		K.NLQMDDFELLCTI
124	560 - 575	661.6400	1981.8982	1981.8986	-0.0004	1	17	4e+003	4		K.NLQMDDFELLCTI
68	575 - 582	512.6700	1023.3254	1023.4920	-0.1665	1	22	7.5e+002	4		R.RANVMDYR.E
126	583 - 600	683.3500	2047.0282	2047.0520	-0.0239	0	29	2.1e+002	1		R.ECNLAEVPTHAVV
89	625 - 633	581.2300	1160.4454	1160.4994	-0.0540	0	23	7.9e+002	4		K.FMMFESQNK.D
206	625 - 638	889.3800	1776.7454	1776.8579	-0.1124	1	48	2.9	1		K.FMMFESQNKDLLF
93	655 - 669	582.9000	1745.6782	1745.9240	-0.2458	1	32	1e+002	1		K.EFLGDKFYTVISS
94	655 - 669	582.9100	1745.7082	1745.9240	-0.2158	1	23	7.9e+002	1		K.EFLGDKFYTVISS
202	655 - 669	873.9800	1745.9454	1745.9240	0.0215	1	50	2.1	1		K.EFLGDKFYTVISS
203	655 - 669	874.0000	1745.9854	1745.9240	0.0615	1	54	0.82	1		K.EFLGDKFYTVISS
78	661 - 669	529.2100	1056.4054	1056.5855	-0.1801	0	36	42	1		K.FYTVISSLK.T
236	670 - 686	1000.4700	1998.9254	1998.8849	0.0405	0	67	0.034	1		K.TCNPSDILQMCSF





Trypsin/LysC mix, digestion 3, measurement 3:

MASCOT Search Results

Protein View: gi|83754919

Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution

Database:	NCBInr
Score:	1345
Nominal mass (M$_r$):	77518
Calculated pI:	6.70
Taxonomy:	Gallus gallus

Sequence similarity is available as an NCBI BLAST search of gi|83754919 against nr.

Search parameters

MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (N-term), Oxidation (M)

Protein sequence coverage: 51%

Matched peptides shown in ***bold red***.

```
  1 APPKSVIRWC TISSPEEKKC NNLRDLTQQE RISLTCVQKA TYLDCIKAIA
 51 NNEADAISLD GGQVFEAGLA PYKLKPIAAE VYEHTEGSTT SYYAVAVVKK
101 GTEFTVNDLQ GKTSCHTGLG RSAGWNIPIG TLIHRGAIEW EGIESGSVEQ
151 AVAKFFSASC VPGATIEQKL CRQCKGDPKT KCARNAPYSG YSGAFHCLKD
201 GKGDVAFVKH TTVNENAPDQ KDEYELLCLD GSRQPVDNYK TCNWARVAAH
251 AVVARDDNKV EDIWSFLSKA QSDFGVDTKS DFHLFGPPGK KDPVLKDLLF
301 KDSAIMLKRV PSLMDSQLYL GFEYYSAIQS MRKDQLTPSP RENRIQWCAV
351 GKDEKSKCDR WSVVSNGDVE CTVVDETKDC IIKIMKGEAD AVALDGGLVY
401 TAGVCGLVPV MAERYDDESQ CSKTDERPAS YFAVAVARKD SNVNWNNLKG
451 KKSCHTAVGR TAGWVIPMGL IHNRTGTCNF DEYFSEGCAP GSPPNSRLCQ
501 LCQGSGGIPP EKCVASSHEK YFGYTGALRC LVEKGDVAFI QHSTVEENTG
551 GKNKADWAKN LQMDDFELLC TDGRRANVMD YRECNLAEVP THAVVVRPEK
601 ANKIRDLLER QEKRFGVNGS EKSKFMMFES QNKDLLFKDL TKCLFKVREG
651 TTYKEFLGDK FYTVISSLKT CNPSDILQMC SFLEGK
```

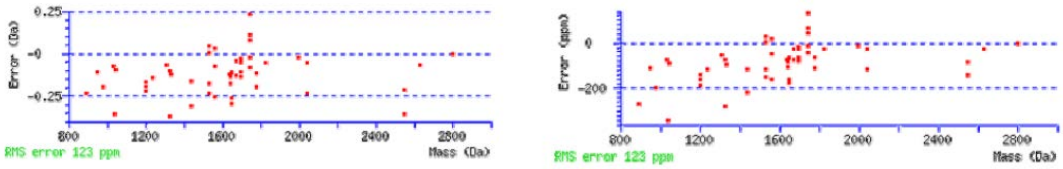
Unformatted sequence string: **686 residues** (for pasting into other applications).

Sort peptides by (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Show predicted peptides also

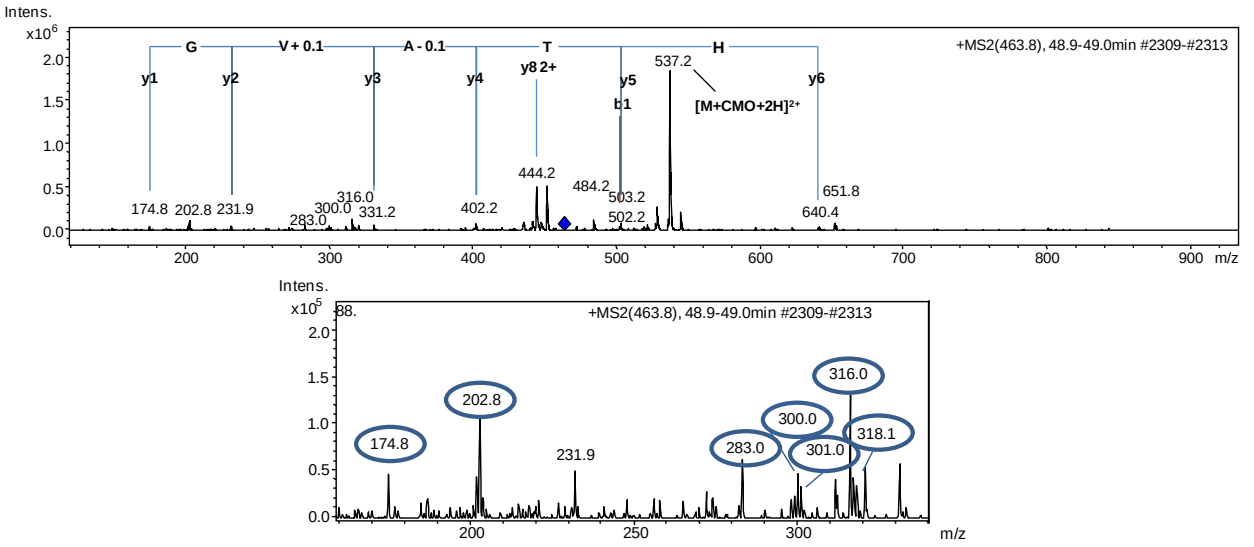
Query	Start – End	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
118	9 – 18	618.7100	1235.4054	1235.5492	-0.1438	0	38	40	1		R.WCTISSPEEK.K
59	32 – 39	474.7100	947.4054	947.5110	-0.1056	0	41	12	1		R.ISLTCVQK.A
62	40 – 47	492.1500	982.2854	982.4794	-0.1939	0	39	18	1		K.ATYLDCIK.A
204	48 – 73	878.7500	2633.2282	2633.2973	-0.0691	0	31	1.5e+002	1		K.AIANNEADAISLI
60	100 – 112	479.4800	1435.4182	1435.7307	-0.3125	1	46	3.5	1		K.KGTEFTVNDLQGI
61	100 – 112	479.5300	1435.5682	1435.7307	-0.1625	1	40	16	1		K.KGTEFTVNDLQGI
120	101 – 112	654.7900	1307.5654	1307.6358	-0.0703	0	74	0.0057	1		K.GTEFTVNDLQGK.
63	122 – 135	512.2100	1533.6082	1533.8416	-0.2334	0	37	25	1		R.SAGWNIPIGTLIH
148	122 – 135	767.8400	1533.6654	1533.8416	-0.1761	0	21	1.3e+003	9		R.SAGWNIPIGTLIH
150	122 – 135	767.9300	1533.8454	1533.8416	0.0039	0	37	28	1		R.SAGWNIPIGTLIH
151	122 – 135	767.9500	1533.8854	1533.8416	0.0439	0	37	28	1		R.SAGWNIPIGTLIH
189	155 – 169	821.3400	1640.6654	1640.7869	-0.1214	0	61	0.15	1		K.FFSASCVPGATIH
196	185 – 199	836.3200	1670.6254	1670.7511	-0.1257	0	67	0.031	1		R.NAPYSGYSGAFHC
80	185 – 199	557.9100	1670.7082	1670.7511	-0.0429	0	16	4.9e+003	2		R.NAPYSGYSGAFHC
69	200 – 209	518.2400	1034.4654	1034.5397	-0.0742	1	31	1.1e+002	1		K.DGKGDVAFVK.H
232	210 – 233	935.4300	2803.2682	2803.2719	-0.0037	1	48	2.4	1		K.HTTVNENAPDQKI
55	247 – 255	447.1500	892.2854	892.5243	-0.2388	0	48	2.2	1		R.VAAHAVVAR.D
81	256 – 269	565.9000	1694.6782	1694.8152	-0.1370	1	32	92	1		R.DDNKVEDIWSFLS
198	256 – 269	848.3600	1694.7054	1694.8152	-0.1097	1	61	0.13	1		R.DDNKVEDIWSFLS
82	256 – 269	565.9300	1694.7682	1694.8152	-0.0470	1	39	16	1		R.DDNKVEDIWSFLS
199	256 – 269	848.4000	1694.7854	1694.8152	-0.0297	1	53	1	1		R.DDNKVEDIWSFLS
40	280 – 290	401.1300	1200.3682	1200.5928	-0.2246	0	25	5.2e+002	1		K.SDFHLFGPPGK.F
41	280 – 290	401.1300	1200.3682	1200.5928	-0.2246	0	30	1.9e+002	1		K.SDFHLFGPPGK.F

42	280 – 290	401.1400	1200.3982	1200.5928	-0.1946	0	24	7e+002	4	K.SDFHLFGPPGK.F
87	280 – 290	601.2200	1200.4254	1200.5928	-0.1673	0	42	15	1	K.SDFHLFGPPGK.F
50	280 – 291	443.7800	1328.3182	1328.6877	-0.3696	1	31	1.2e+002	1	K.SDFHLFGPPGKK.
51	280 – 291	443.8700	1328.5882	1328.6877	-0.0996	1	37	33	1	K.SDFHLFGPPGKK.
70	333 – 341	521.1100	1040.2054	1040.5614	-0.3560	1	39	18	1	R.KDQLTPSPR.E
123	345 – 355	667.2700	1332.5254	1332.6496	-0.1242	1	50	1.8	1	R.IQWCAVGKDEK.S
75	424 – 438	551.5200	1651.5382	1651.8318	-0.2936	0	44	6.5	1	K.TDERPASYFAVAV
76	424 – 438	551.5300	1651.5682	1651.8318	-0.2636	0	50	1.6	1	K.TDERPASYFAVAV
77	424 – 438	551.5800	1651.7182	1651.8318	-0.1136	0	51	1.2	1	K.TDERPASYFAVAV
192	424 – 438	826.8700	1651.7254	1651.8318	-0.1064	0	36	40	1	K.TDERPASYFAVAV
71	461 – 474	522.2000	1563.5782	1563.8344	-0.2562	0	30	1.4e+002	2	R.TAGWVIPMGLIHN
72	461 – 474	522.2600	1563.7582	1563.8344	-0.0762	0	34	53	1	R.TAGWVIPMGLIHN
179	461 – 474	782.9400	1563.8654	1563.8344	0.0311	0	24	6e+002	2	R.TAGWVIPMGLIHN
250	475 – 497	1275.3400	2548.6654	2549.0224	-0.3569	0	61	0.1	1	R.TGTCNFDEYFSEC
200	475 – 497	850.6100	2548.8082	2549.0224	-0.2142	0	34	65	1	R.TGTCNFDEYFSEC
190	498 – 512	822.3100	1642.6054	1642.7807	-0.1753	0	49	2.2	1	R.LCQLCQGSGGIPI
191	498 – 512	822.3300	1642.6454	1642.7807	-0.1353	0	37	31	1	R.LCQLCQGSGGIPI
73	521 – 529	524.2200	1046.4254	1046.5185	-0.0931	0	48	2.1	1	K.YFGYTGALR.C
214	560 – 574	913.8800	1825.7454	1825.7975	-0.0520	0	61	0.12	1	K.NLQMDDFELLCTI
125	583 – 600	683.2800	2046.8182	2047.0520	-0.2339	0	41	13	1	R.ECNLAEVPTHAVV
126	583 – 600	683.3400	2046.9982	2047.0520	-0.0539	0	28	2.5e+002	8	R.ECNLAEVPTHAVV
205	625 – 638	889.3400	1776.6654	1776.8579	-0.1924	1	47	3.9	1	K.FMMFESQNKDLLI
206	625 – 638	889.3800	1776.7454	1776.8579	-0.1124	1	41	15	1	K.FMMFESQNKDLLI
201	655 – 669	873.9300	1745.8454	1745.9240	-0.0785	1	66	0.052	1	K.EFLGDKFYTVISS
202	655 – 669	873.9600	1745.9054	1745.9240	-0.0185	1	53	1	1	K.EFLGDKFYTVISS
203	655 – 669	874.0100	1746.0054	1745.9240	0.0815	1	69	0.021	1	K.EFLGDKFYTVISS
85	655 – 669	583.0200	1746.0382	1745.9240	0.1142	1	20	1.5e+003	2	K.EFLGDKFYTVISS
86	655 – 669	583.0600	1746.1582	1745.9240	0.2342	1	30	1.6e+002	1	K.EFLGDKFYTVISS
238	670 – 686	1000.4400	1998.8654	1998.8849	-0.0195	0	95	4.8e-005	1	K.TCNPSDILQMCSI



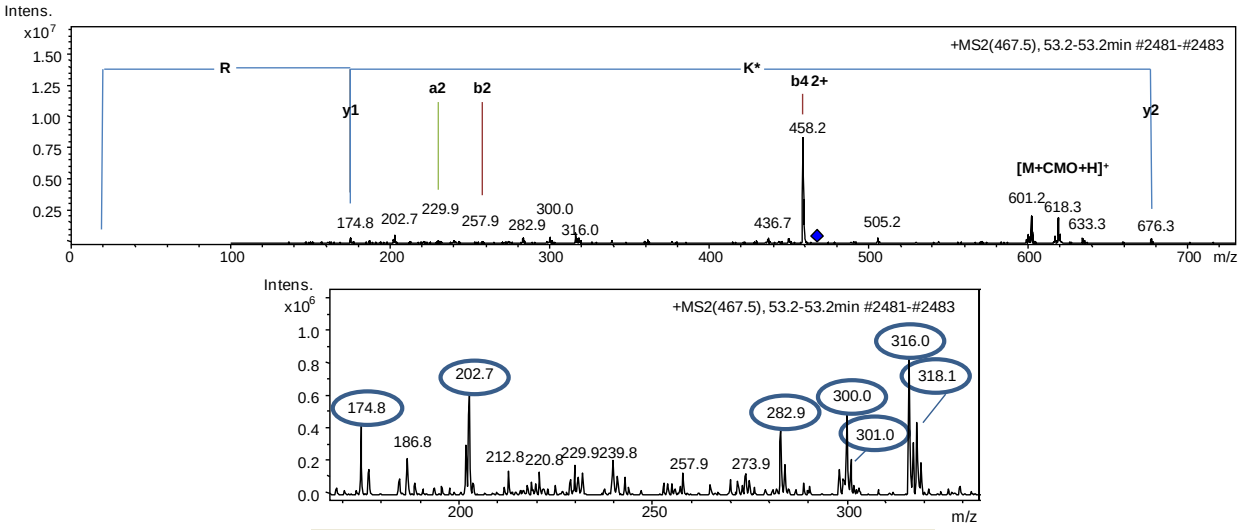
7.2 MS/MS spectra of identified modified peptides

MS/MS spectra of identified modified peptides are collected in this chapter. The top spectrum shows identified peptide fragment ions. The bottom spectrum is a zoomed in spectrum, which displays the reporter ions for verification that the peptide was modified.



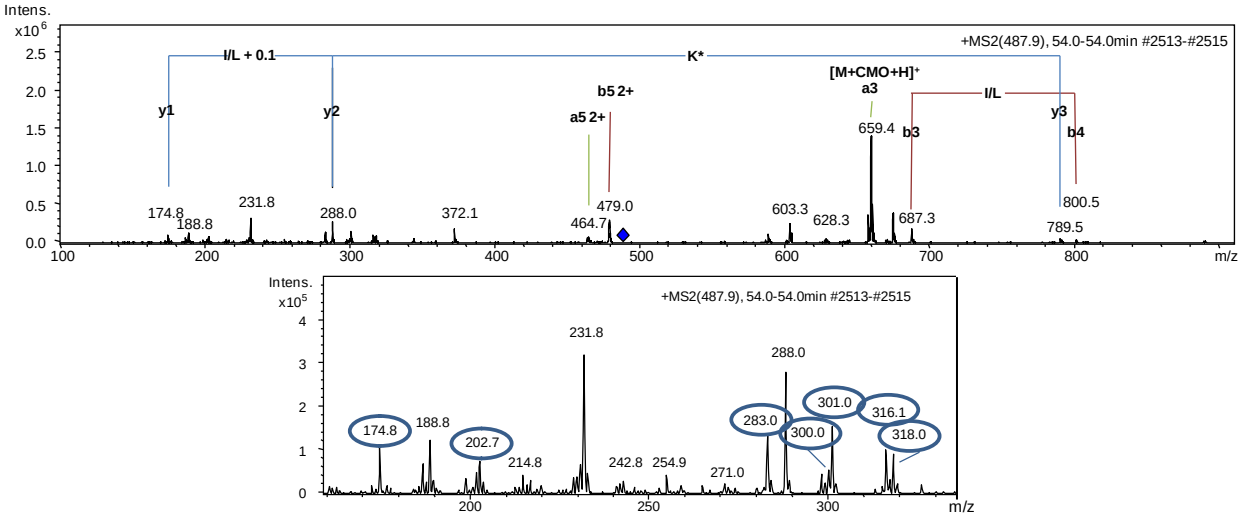
	K	S	C	H	T	A	V	G	R	Lys	Ser	Cys	His	Thr	Ala	Val	Gly	Arg
Ion	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
a	K*	S	C	H	T	A	V	G	R	474.260	561.292	721.323	858.381	959.429	1030.466	1129.535	1186.556	1342.657
b	K*	S	C	H	T	A	V	G	R	502.255	589.287	749.317	886.376	987.424	1058.461	1157.530	1214.551	1370.652
y	K*	S	C	H	T	A	V	G	R	175.119	232.140	331.209	402.246	503.294	640.353	800.383	887.415	1388.663
	9	8	7	6	5	4	3	2	1	Arg	Gly	Val	Ala	Thr	His	Cys	Ser	Lys

Figure 58 MS/MS spectrum of 463.5^{3+} K.KSCHTAVGR.T, 1: ZEN-CMO (K), 3: Carbamidomethyl (C), MW =1387.7 Da



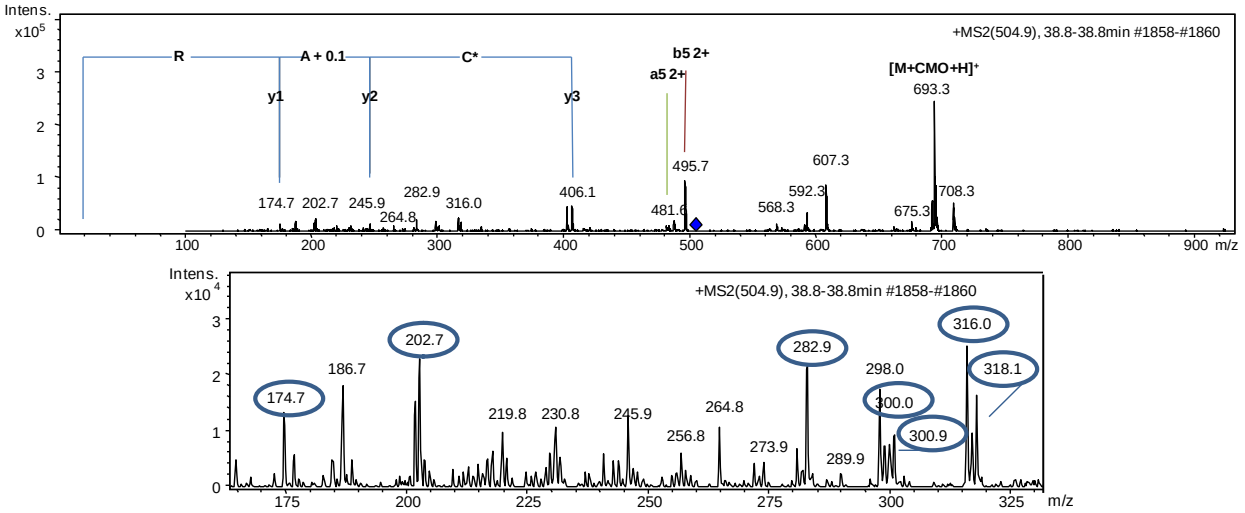
	Q	E	K	R	Gln	Glu	Lys	Arg
Ion	1	2	3	4	1	2	3	4
a	Q	E	K*	R	101.071	230.114	731.361	887.462
b	Q	E	K*	R	129.066	258.108	759.356	915.457
y	Q	E	K*	R	175.119	676.366	805.409	933.468
	4	3	2	1	Arg	Lys	Glu	Gln

Figure 59 MS/MS spectrum of 467.2^{2+} R.QEKR.F, 3: ZEN-CMO (K), MW = 932.5 Da



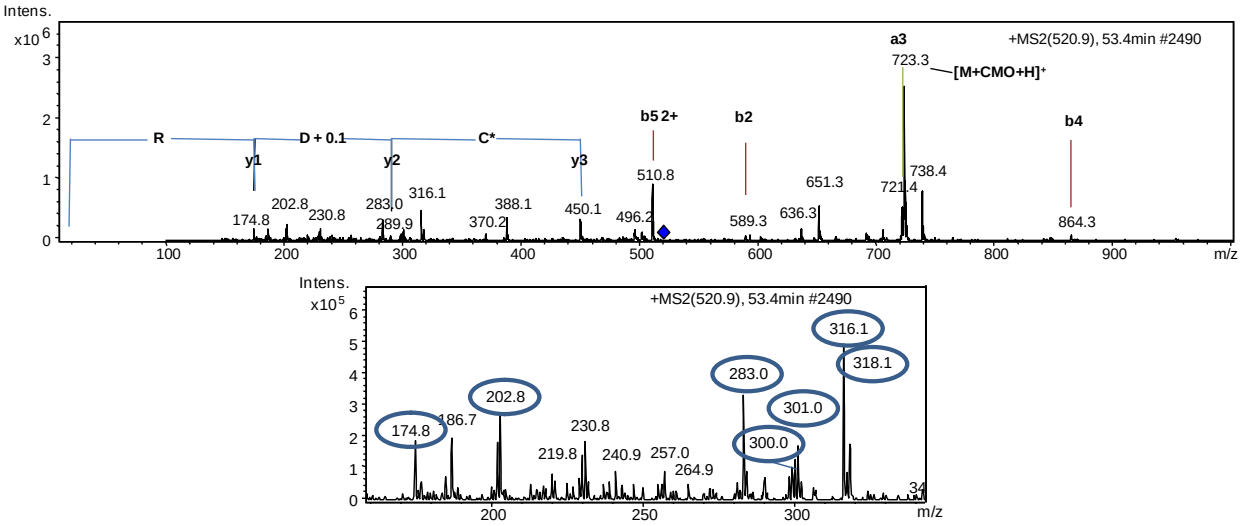
	A	N	K	I	R	Ala	Asn	Lys	Ile	Arg
Ion	1	2	3	4	5	1	2	3	4	5
a	A	N	K*	I	R	44.049	158.092	659.340	772.424	928.525
b	A	N	K*	I	R	72.044	186.087	687.335	800.419	956.520
y	A	N	K*	I	R	175.119	288.203	789.451	903.493	974.531
	5	4	3	2	1	Arg	Ile	Lys	Asn	Ala

Figure 60 MS/MS spectrum of 487.8^{2+} K.ANKIR.D 3: ZEN-CMO (K), MW=973.5 Da



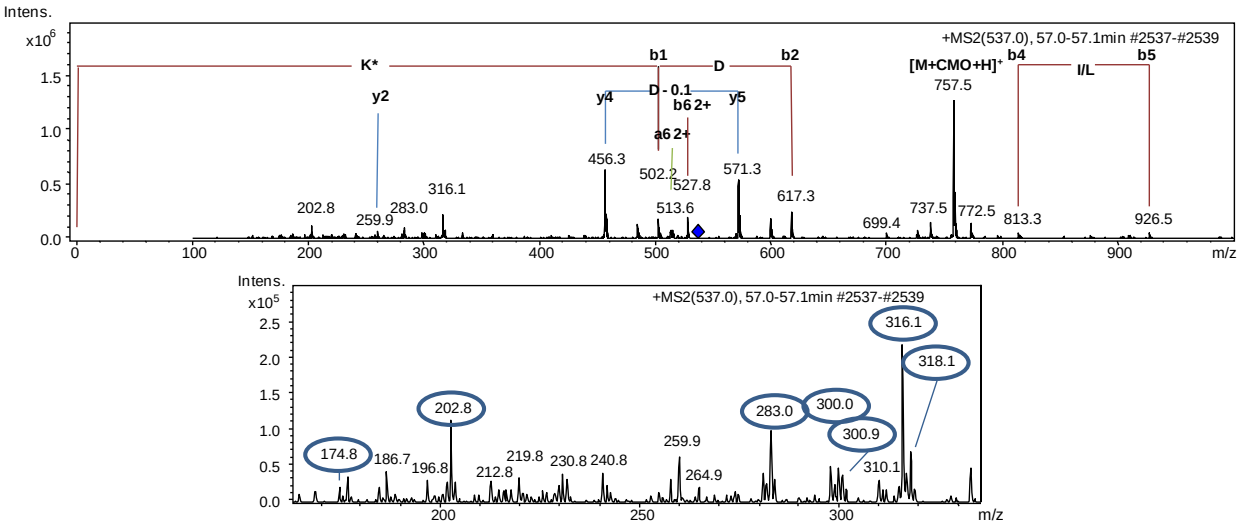
	T	K	C	A	R	Thr	Lys	Cys	Ala	Arg
Ion	1	2	3	4	5	1	2	3	4	5
a	T	K*	C	A	R	74.060	575.308	735.338	806.375	962.476
b	T	K*	C	A	R	102.055	603.302	763.333	834.370	990.471
y	T	K*	C	A	R	175.119	246.156	406.187	907.434	1008.482
	5	4	3	2	1	Arg	Ala	Cys	Lys	Thr

Figure 61 MS/MS spectrum of 504.7^{2+} K.TKCAR.N 2: ZEN-CMO (K), 3: Carbamidomethyl (C), MW=1007.5 Da



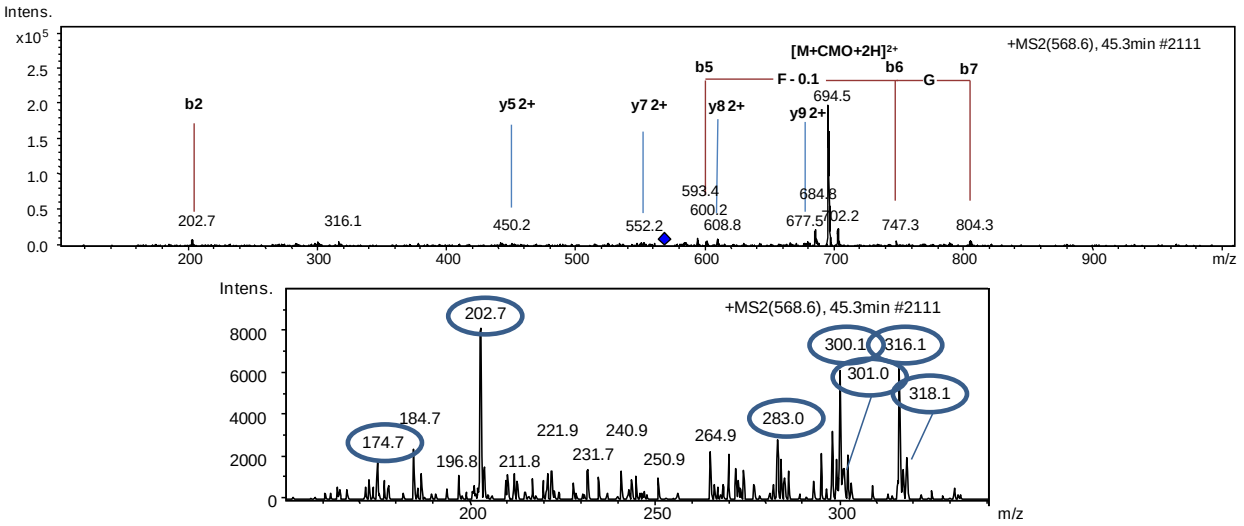
	S	K	C	D	R	Ser	Lys	Cys	Asp	Arg
Ion	1	2	3	4	5	1	2	3	4	5
a	S	K*	C	D	R	60.044	561.292	721.323	836.349	992.451
b	S	K*	C	D	R	88.039	589.287	749.317	864.344	1020.446
y	S	K*	C	D	R	175.119	290.146	450.177	951.424	1038.456
	5	4	3	2	1	Arg	Asp	Cys	Lys	Ser

Figure 62 MS/MS spectrum of 519.7^{2+} K.SKCDR.W 2: ZEN-CMO (K), 3: Carbamidomethyl (C), MW=1037.4 Da



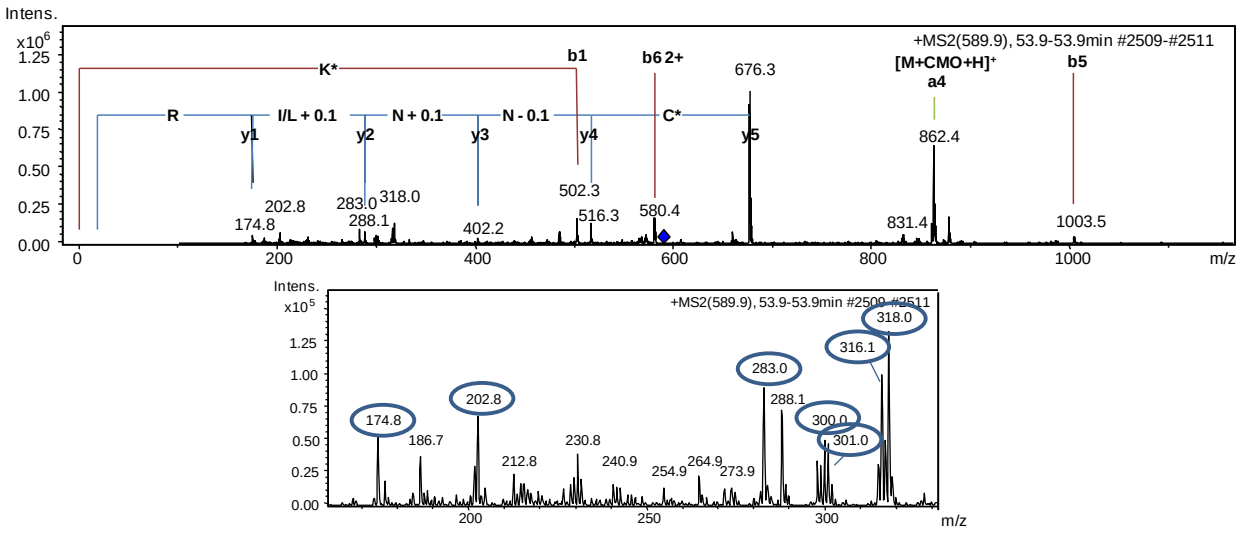
	K	D	P	V	L	K	Lys	Asp	Pro	Val	Leu	Lys
Ion	1	2	3	4	5	6	1	2	3	4	5	6
a	K*	D	P	V	L	K	474.260	589.287	686.340	785.408	898.492	1026.587
b	K*	D	P	V	L	K	502.255	617.282	714.334	813.403	926.487	1054.582
y	K*	D	P	V	L	K	147.113	260.197	359.265	456.318	571.345	1072.592
	6	5	4	3	2	1	Lys	Leu	Val	Pro	Asp	Lys

Figure 63 MS/MS spectrum of 536.8^{2+} K.KDPVLK.D 1: ZEN-CMO (K), MW=1071.6 Da



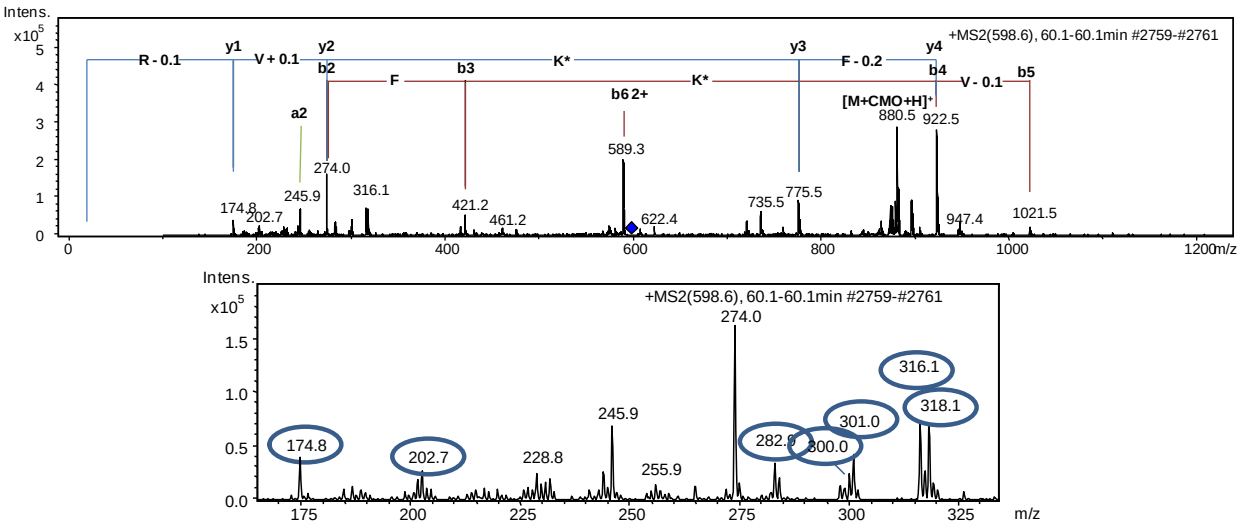
	S	D	F	H	L	F	G	P	P	G	K	K	Ser	Asp	Phe	His	Leu	Phe	Gly	Pro	Pro	Gly	Lys	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
a	S	D	F	H	L	F	G	P	P	G	K*	K	60.044	175.071	322.140	459.199	572.283	719.351	776.373	873.425	970.478	1027.500	1528.747	1656.842
b	S	D	F	H	L	F	G	P	P	G	K*	K	88.039	203.066	350.135	487.194	600.278	747.346	804.368	901.420	998.473	1055.495	1556.742	1684.837
y	S	D	F	H	L	F	G	P	P	G	K*	K	147.113	648.360	705.382	802.435	899.487	956.509	1103.577	1216.661	1353.720	1500.789	1615.816	1702.848
	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Lys	Gly	Pro	Pro	Gly	Phe	Leu	His	Phe	Asp	Ser

Figure 64 MS/MS spectrum of 568.3^{3+} K.SDFHLFGPPGKK.D 11: ZEN-CMO (K), MW = 1701.8 Da



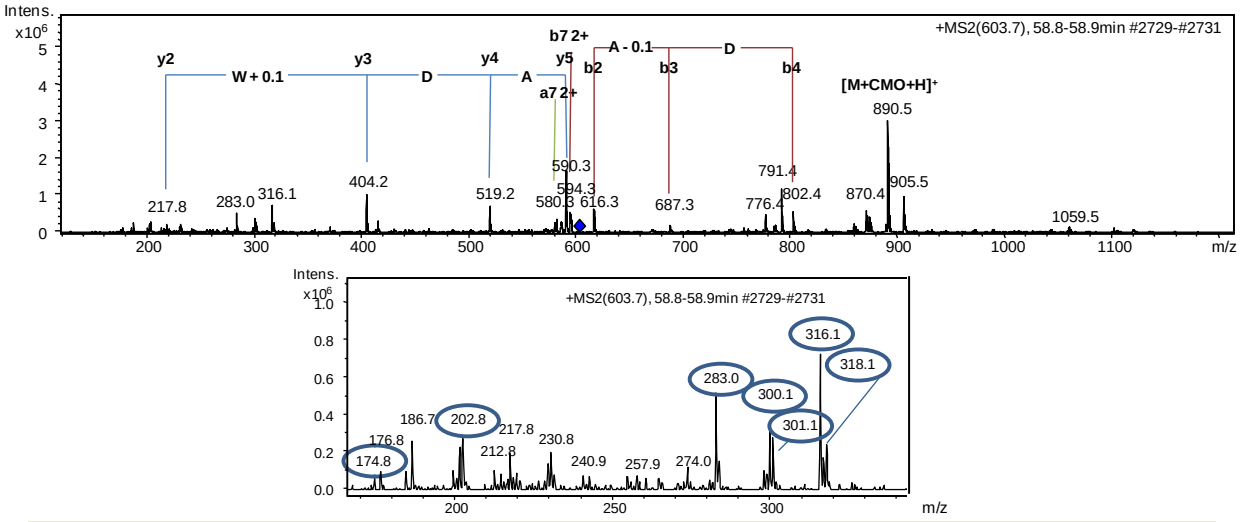
	K	C	N	N	L	R	Lys	Cys	Asn	Asn	Leu	Arg
Ion	1	2	3	4	5	6	1	2	3	4	5	6
a	K*	C	N	N	L	R	474.260	634.291	748.333	862.376	975.460	1131.562
b	K*	C	N	N	L	R	502.255	662.285	776.328	890.371	1003.455	1159.556
y	K*	C	N	N	L	R	175.119	288.203	402.246	516.289	676.320	1177.567
	6	5	4	3	2	1	Arg	Leu	Asn	Asn	Cys	Lys

Figure 65 MS/MS spectrum of 589.3^{2+} K.KCNNLR.D 1: ZEN-CMO (K), 2: Carbamidomethyl (C), MW=1176.6 Da



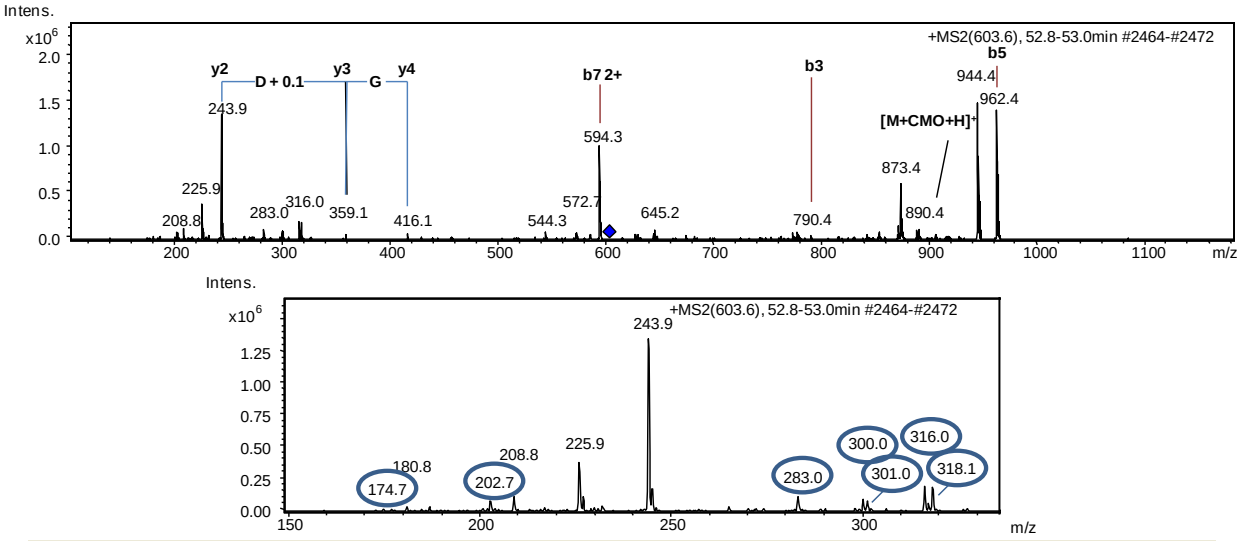
	C	L	F	K	V	R	Cys	Leu	Phe	Lys	Val	Arg
Ion	1	2	3	4	5	6	1	2	3	4	5	6
a	C	L	F	K*	V	R	133.043	246.127	393.195	894.443	993.511	1149.613
b	C	L	F	K*	V	R	161.038	274.122	421.190	922.438	1021.506	1177.607
y	C	L	F	K*	V	R	175.119	274.187	775.435	922.503	1035.587	1195.618
	6	5	4	3	2	1	Arg	Val	Lys	Phe	Leu	Cys

Figure 66 MS/MS spectrum of 598.3^{2+} K.CLFKVR.E 1: Carbamidomethyl, 4: ZEN-CMO (K), MW=1194.6



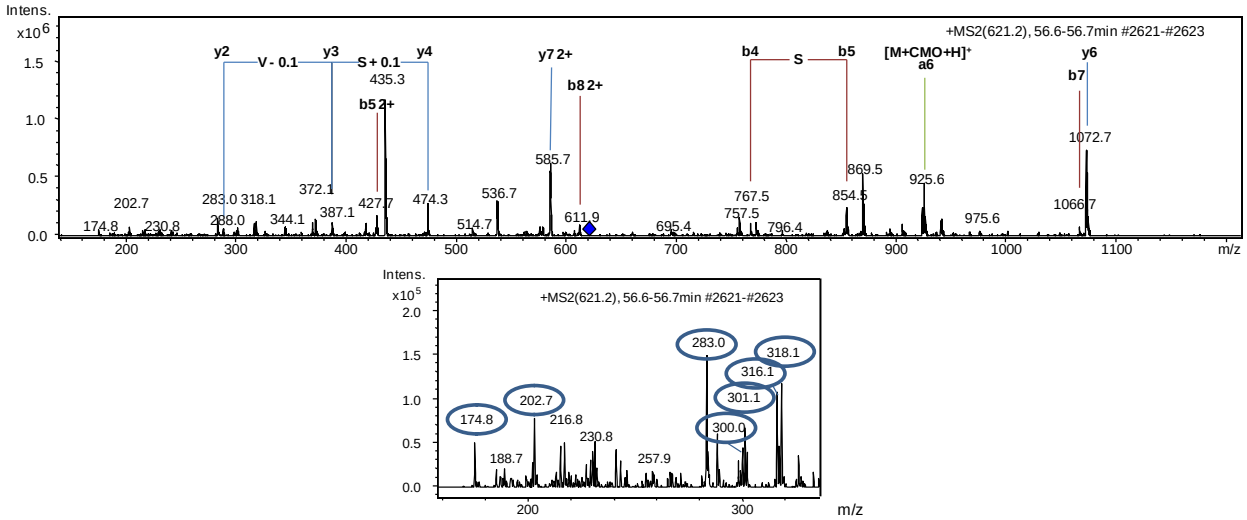
	N	K	A	D	W	A	K	Asn	Lys	Ala	Asp	Trp	Ala	Lys
Ion	1	2	3	4	5	6	7	1	2	3	4	5	6	7
a	N	K*	A	D	W	A	K	87.055	588.303	659.340	774.367	960.446	1031.483	1159.578
b	N	K*	A	D	W	A	K	115.050	616.298	687.335	802.362	988.441	1059.478	1187.573
y	N	K*	A	D	W	A	K	147.113	218.150	404.229	519.256	590.293	1091.541	1205.584
	7	6	5	4	3	2	1	Lys	Ala	Trp	Asp	Ala	Lys	Asn

Figure 67 MS/MS spectrum of 603.3^{2+} K.NKADWAK.N 2: ZEN-CMO (K), MW=1204.6 Da



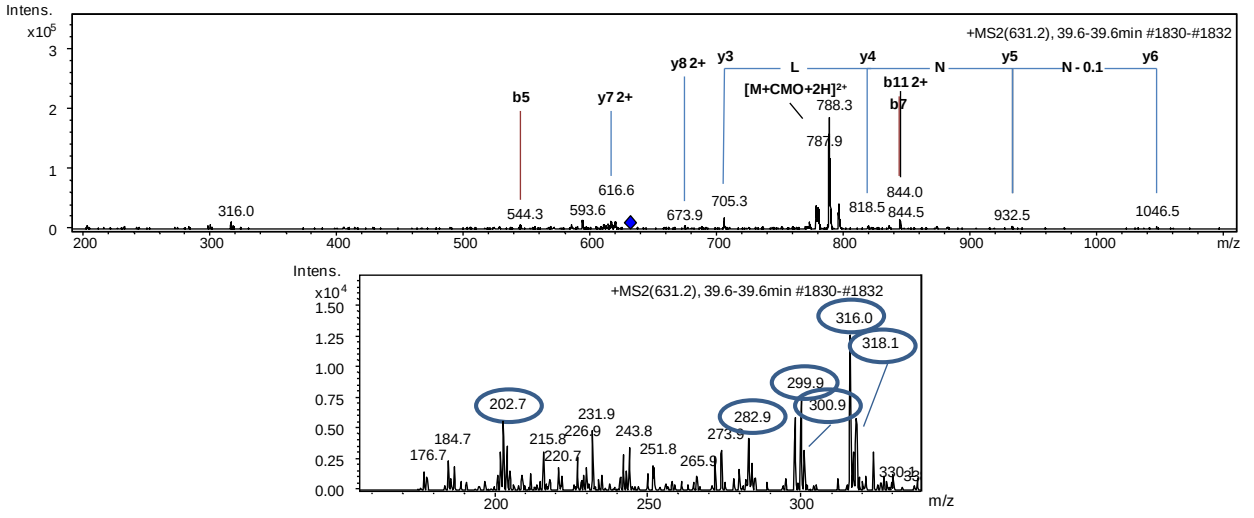
	Q	C	K	G	D	P	K	Gln	Cys	Lys	Gly	Asp	Pro	Lys
Ion	1	2	3	4	5	6	7	1	2	3	4	5	6	7
a	Q	C	K*	G	D	P	K	101.071	261.102	762.349	819.371	934.397	1031.450	1159.545
b	Q	C	K*	G	D	P	K	129.066	289.097	790.344	847.365	962.392	1059.445	1187.540
y	Q	C	K*	G	D	P	K	147.113	244.166	359.193	416.214	917.461	1077.492	1205.551
	7	6	5	4	3	2	1	Lys	Pro	Asp	Gly	Lys	Cys	Gln

Figure 68 MS/MS spectrum of 603.3^{2+} R.QCKGDPK.T 2: Carbamidomethyl (C), 3: ZEN-CMO (K), MW=1204.5 Da



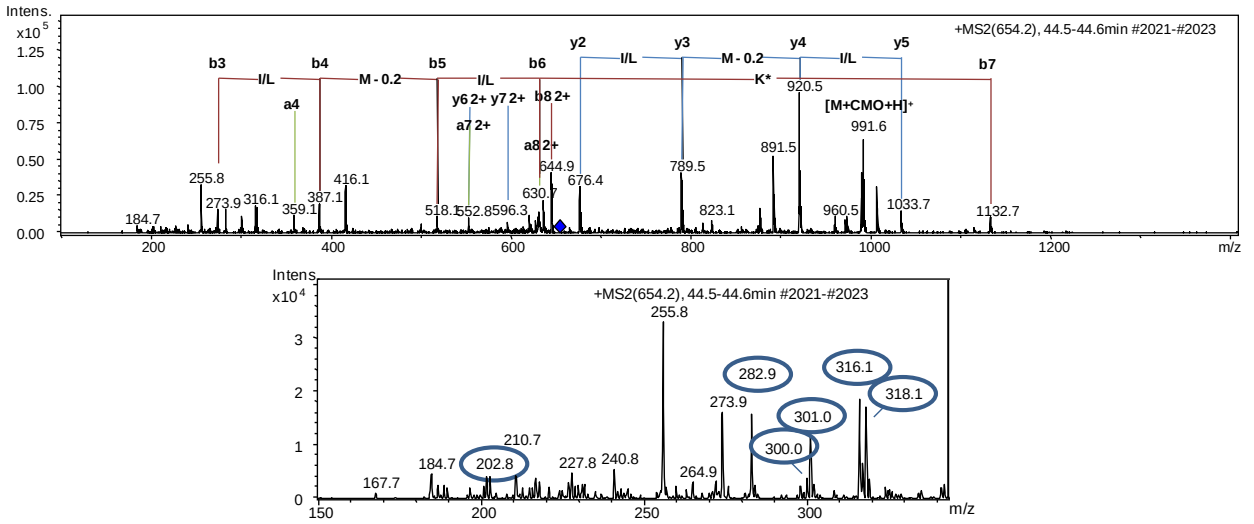
	A	P	P	K	S	V	I	R	Ala	Pro	Pro	Lys	Ser	Val	Ile	Arg
Ion	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
a	A	P	P	K*	S	V	I	R	44.049	141.102	238.155	739.403	826.435	925.503	1038.587	1194.688
b	A	P	P	K*	S	V	I	R	72.044	169.097	266.150	767.397	854.429	953.498	1066.582	1222.683
y	A	P	P	K*	S	V	I	R	175.119	288.203	387.271	474.303	975.551	1072.604	1169.656	1240.694
	8	7	6	5	4	3	2	1	Arg	Ile	Val	Ser	Lys	Pro	Pro	Ala

Figure 69 MS/MS spectrum of 620.8^{2+} -.APPKSVIR.W 4: ZEN-CMO (K), MW=1239.7 Da



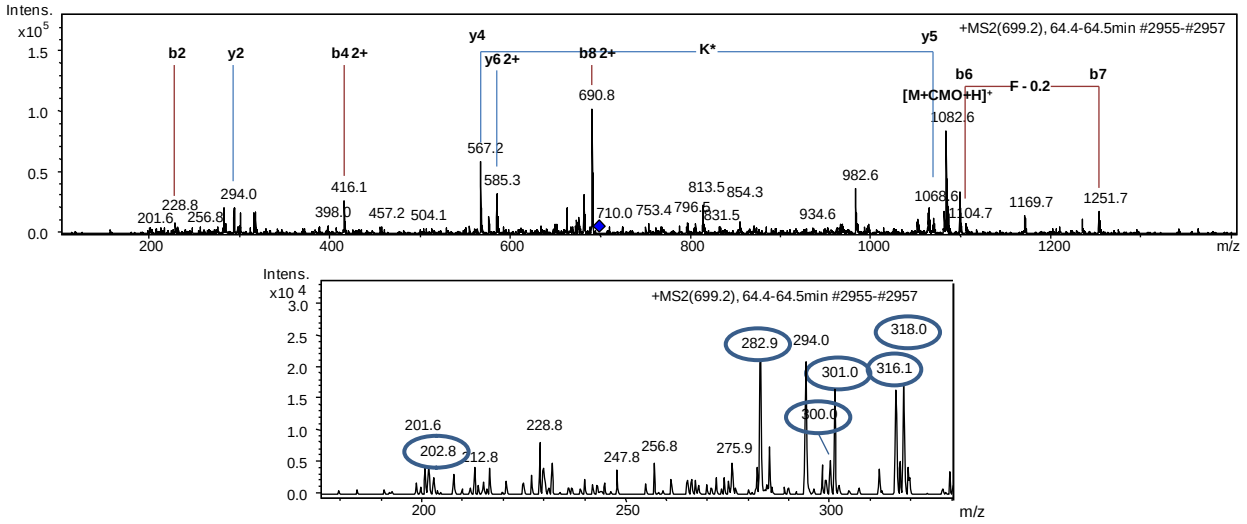
	K	D	S	N	V	N	W	N	N	L	K	G	K	Lys	Asp	Ser	Asn	Val	Asn	Trp	Asn	Asn	Leu	Lys	Gly	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9	10	11	12	13
a	K	D	S	N	V	N	W	N	N	L	K*	G	K	101.107	216.134	303.166	417.209	516.278	630.321	816.400	930.443	1044.486	1157.570	1658.817	1715.839	1843.934
b	K	D	S	N	V	N	W	N	N	L	K*	G	K	129.102	244.129	331.161	445.204	544.273	658.315	844.395	958.438	1072.481	1185.565	1686.812	1743.834	1871.929
y	K	D	S	N	V	N	W	N	N	L	K*	G	K	147.113	204.134	705.382	818.466	932.509	1046.552	1232.631	1346.674	1445.742	1559.785	1646.817	1761.844	1889.939
	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Gly	Lys	Leu	Asn	Asn	Trp	Asn	Val	Asn	Ser	Asp	Lys

Figure 70 MS/MS spectrum of 630.6^{3+} R.KDSNVNWNNLKGK.K 11: ZEN-CMO (K), MW=1888.9 Da



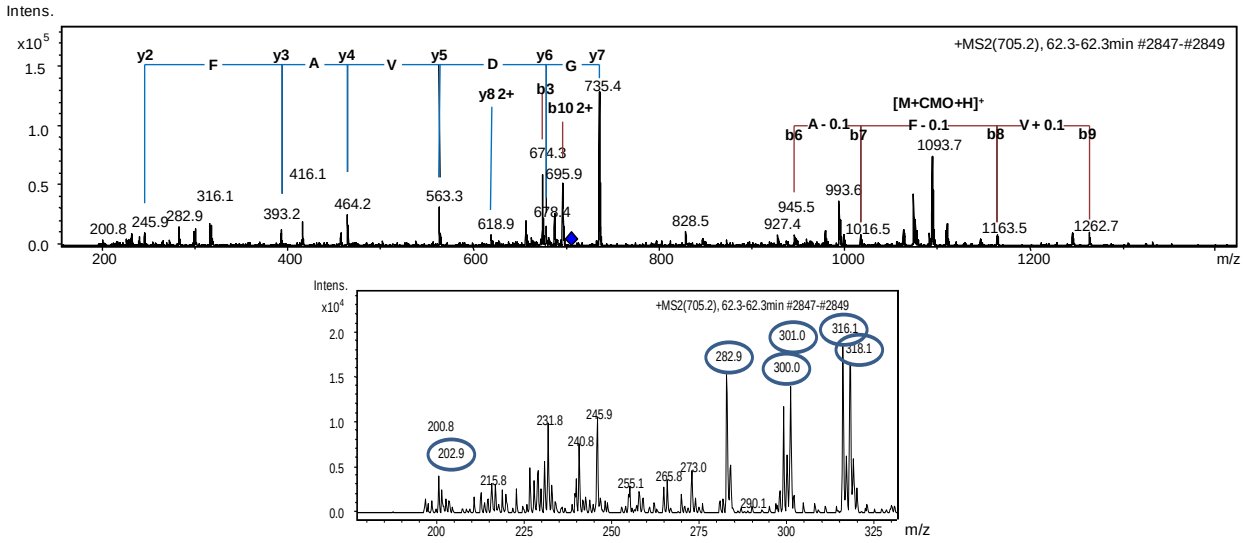
	D	S	A	I	M	L	K	R	Asp	Ser	Ala	Ile	Met	Leu	Lys	Arg
Ion	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
a	D	S	A	I	M	L	K*	R	88.039	175.071	246.108	359.193	490.233	603.317	1104.565	1260.666
b	D	S	A	I	M	L	K*	R	116.034	203.066	274.103	387.187	518.228	631.312	1132.559	1288.661
y	D	S	A	I	M	L	K*	R	175.119	676.366	789.451	920.491	1033.575	1104.612	1191.644	1306.671
	8	7	6	5	4	3	2	1	Arg	Lys	Leu	Met	Ile	Ala	Ser	Asp

Figure 71 MS/MS spectrum of 653.8^{2+} K.DSAIMLKR.V 7: ZEN-CMO (K), MW= 1305.7 Da



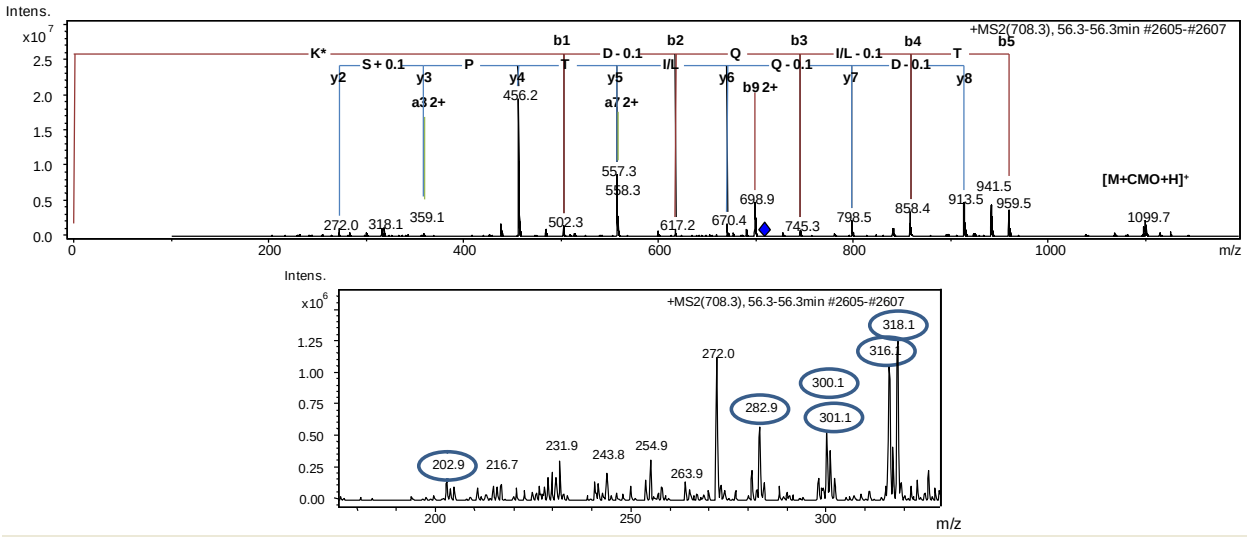
	D	L	T	K	C	L	F	K	Asp	Leu	Thr	Lys	Cys	Leu	Phe	Lys
Ion	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
a	D	L	T	K*	C	L	F	K	88.039	201.123	302.171	803.419	963.449	1076.533	1223.602	1351.697
b	D	L	T	K*	C	L	F	K	116.034	229.118	330.166	831.413	991.444	1104.528	1251.597	1379.692
y	D	L	T	K*	C	L	F	K	147.113	294.181	407.265	567.296	1068.543	1169.591	1282.675	1397.702
	8	7	6	5	4	3	2	1	Lys	Phe	Leu	Cys	Lys	Thr	Leu	Asp

Figure 72 MS/MS spectrum of 699.3^{2+} K.DLTKCLFK.V 4: ZEN-CMO (K), 5: Carbamidomethyl (C), MW=1396.7 Da



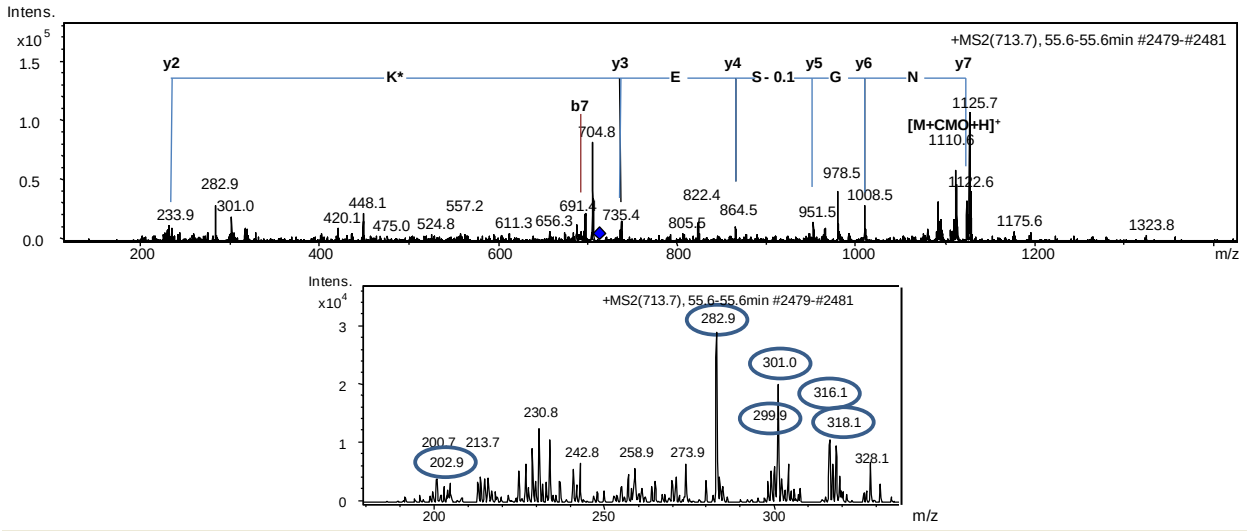
	D	G	K	G	D	V	A	F	V	K	Asp	Gly	Lys	Gly	Asp	Val	Ala	Phe	Val	Lys
Ion	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
a	D	G	K*	G	D	V	A	F	V	K	88.039	145.061	646.308	703.330	818.357	917.425	988.462	1135.531	1234.599	1362.694
b	D	G	K*	G	D	V	A	F	V	K	116.034	173.056	674.303	731.325	846.352	945.420	1016.457	1163.526	1262.594	1390.689
y	D	G	K*	G	D	V	A	F	V	K	147.113	246.181	393.250	464.287	563.355	678.382	735.404	1236.651	1293.673	1408.699
	10	9	8	7	6	5	4	3	2	1	Lys	Val	Phe	Ala	Val	Asp	Gly	Lys	Gly	Asp

Figure 73 MS/MS spectrum of 704.8^{2+} K.DGKGDVAFVK.H 3: ZEN-CMO (K), MW=1407.7 Da



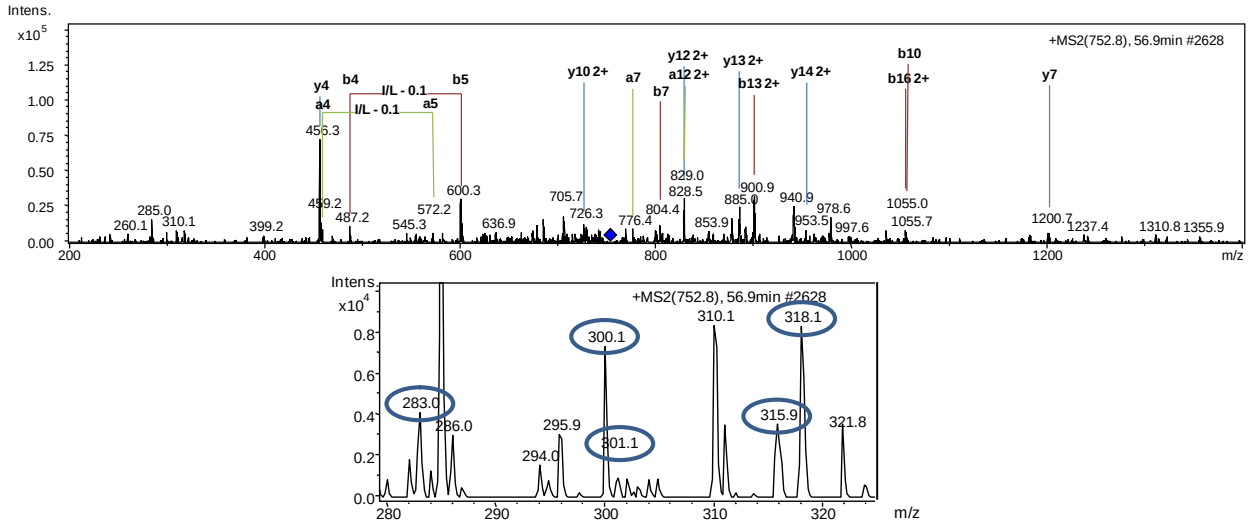
	K	D	Q	L	T	P	S	P	R	Lys	Asp	Gln	Leu	Thr	Pro	Ser	Pro	Arg
Ion	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
a	K*	D	Q	L	T	P	S	P	R	474.260	589.287	717.345	830.429	931.477	1028.530	1115.562	1212.615	1368.716
b	K*	D	Q	L	T	P	S	P	R	502.255	617.282	745.340	858.424	959.472	1056.525	1143.557	1240.610	1396.711
y	K*	D	Q	L	T	P	S	P	R	175.119	272.172	359.204	456.257	557.304	670.388	798.447	913.474	1414.721
	9	8	7	6	5	4	3	2	1	Arg	Pro	Ser	Pro	Thr	Leu	Gln	Asp	Lys

Figure 74 MS/MS spectrum of 707.9^{2+} R.KDQLTPSPR.E 1: ZEN-CMO (K), MW=1413.7 Da



	F	G	V	N	G	S	E	K	S	K	Phe	Gly	Val	Asn	Gly	Ser	Glu	Lys	Ser	Lys
Ion	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	
a	F	G	V	N	G	S	E	K*	S	K	120.081	177.102	276.171	390.214	447.235	534.267	663.310	1164.557	1251.589	1379.684
b	F	G	V	N	G	S	E	K*	S	K	148.076	205.097	304.166	418.208	475.230	562.262	691.305	1192.552	1279.584	1407.679
y	F	G	V	N	G	S	E	K*	S	K	147.113	234.145	735.392	864.435	951.467	1008.488	1122.531	1221.600	1278.621	1425.690
	10	9	8	7	6	5	4	3	2	1	Lys	Ser	Lys	Glu	Ser	Gly	Asn	Val	Gly	Phe

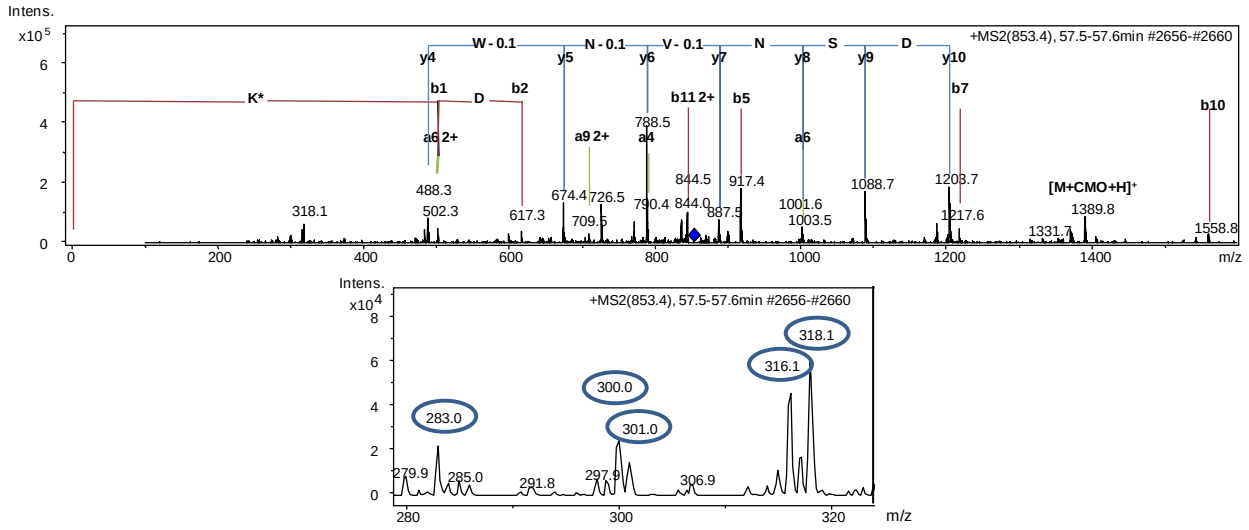
Figure 75 MS/MS spectrum of 713.3^{2+} R.FGVNGSEKSK.F 8: ZEN-CMO (K), MW= 1424.7 Da



	S	D	F	H	L	F	G	P	P	G	K	K	D	P	V	L	K	Ser	Asp	Phe	His	Leu	Phe	Gly
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	1	2	3	4	5	6	7
a	S	D	F	H	L	F	G	P	P	G	K*	K	D	P	V	L	K	60.044	175.071	322.140	459.199	572.283	719.351	776.373
b	S	D	F	H	L	F	G	P	P	G	K*	K	D	P	V	L	K	88.039	203.066	350.135	487.194	600.278	747.346	804.368
y	S	D	F	H	L	F	G	P	P	G	K*	K	D	P	V	L	K	147.113	260.197	359.265	456.318	571.345	699.440	1200.687
	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Leu	Val	Pro	Asp	Lys	Lys

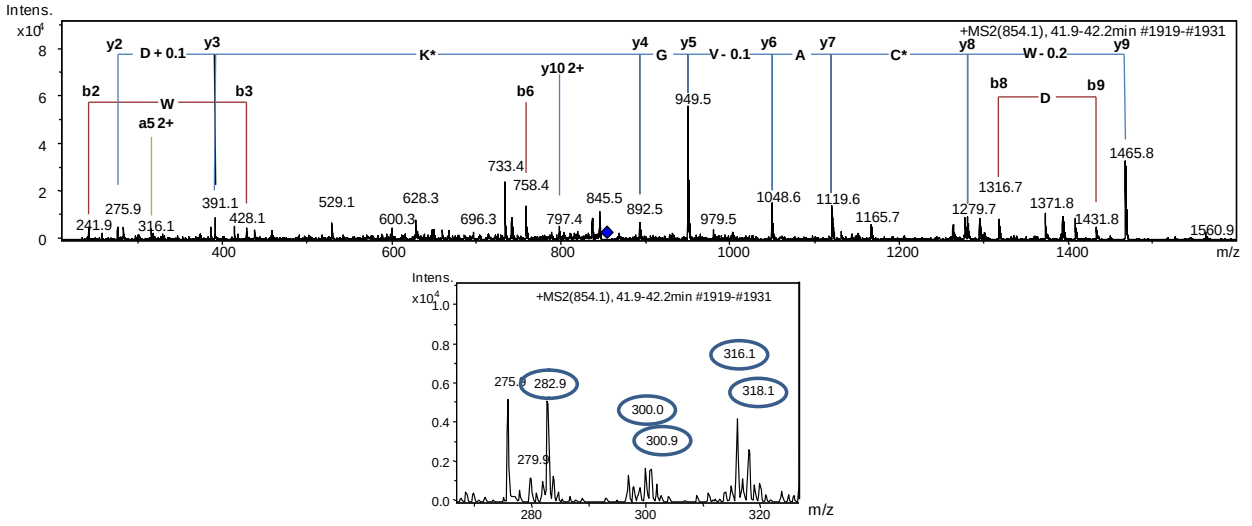
	Pro	Pro	Gly	Lys	Lys	Asp	Pro	Val	Leu	Lys
	8	9	10	11	12	13	14	15	16	17
	873.425	970.478	1027.500	1528.747	1656.842	1771.869	1868.922	1967.990	2081.074	2209.169
	901.420	998.473	1055.495	1556.742	1684.837	1799.864	1896.917	1995.985	2109.069	2237.164
	1257.709	1354.762	1451.814	1508.836	1655.904	1768.988	1906.047	2053.116	2168.143	2255.175
	Gly	Pro	Pro	Gly	Phe	Leu	His	Phe	Asp	Ser

Figure 76 MS/MS spectrum of 752.4^{3+} K.SDFHLFGPPGKKDPVLK.D 11: ZEN-CMO (K), MW=2254. 2 Da



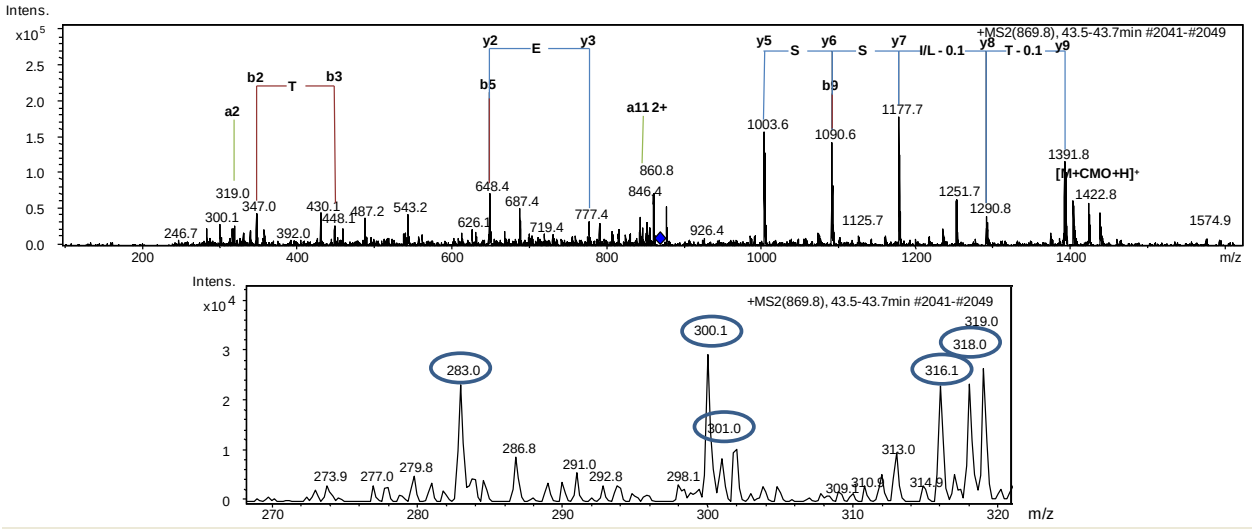
	K	D	S	N	V	N	W	N	N	L	K	Lys	Asp	Ser	Asn	Val	Asn	Trp	Asn	Asn	Leu	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	K*	D	S	N	V	N	W	N	N	L	K	474.260	589.287	676.319	790.362	889.430	1003.473	1189.552	1303.595	1417.638	1530.722	1658.817
b	K*	D	S	N	V	N	W	N	N	L	K	502.255	617.282	704.314	818.357	917.425	1031.468	1217.547	1331.590	1445.633	1558.717	1686.812
y	K*	D	S	N	V	N	W	N	N	L	K	147.113	260.197	374.240	488.283	674.362	788.405	887.473	1001.516	1088.548	1203.575	1704.823
	11	10	9	8	7	6	5	4	3	2	1	Lys	Leu	Asn	Asn	Trp	Asn	Val	Asn	Ser	Asp	Lys

Figure 77 MS/MS spectrum of 852.9^{2+} R.KDSNVNWNNLK.G 1: ZEN-CMO (K), MW = 1703.8 Da



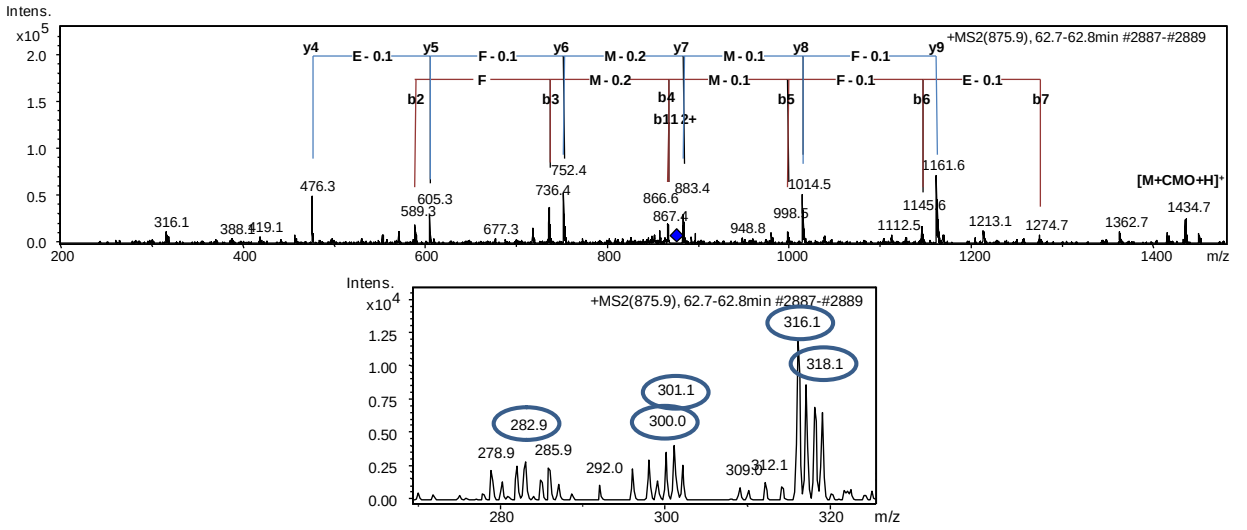
	I	Q	W	C	A	V	G	K	D	E	K	Ile	Gln	Trp	Cys	Ala	Val	Gly	Lys	Asp	Glu	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	I	Q	W	C	A	V	G	K*	D	E	K	86.096	214.155	400.234	560.265	631.302	730.370	787.392	1288.639	1403.666	1532.709	1660.804
b	I	Q	W	C	A	V	G	K*	D	E	K	114.091	242.150	428.229	588.260	659.297	758.365	815.387	1316.634	1431.661	1560.704	1688.799
y	I	Q	W	C	A	V	G	K*	D	E	K	147.113	276.155	391.182	892.430	949.451	1048.520	1119.557	1279.587	1465.667	1593.725	1706.809
	11	10	9	8	7	6	5	4	3	2	1	Lys	Glu	Asp	Lys	Gly	Val	Ala	Cys	Trp	Gln	Ile

Figure 78 MS/MS spectrum of 853.9^{2+} R.IQWCAVGKDEK.S 4: Carbamidomethyl (C), 8: ZEN-CMO (K), MW= 1705.8 Da



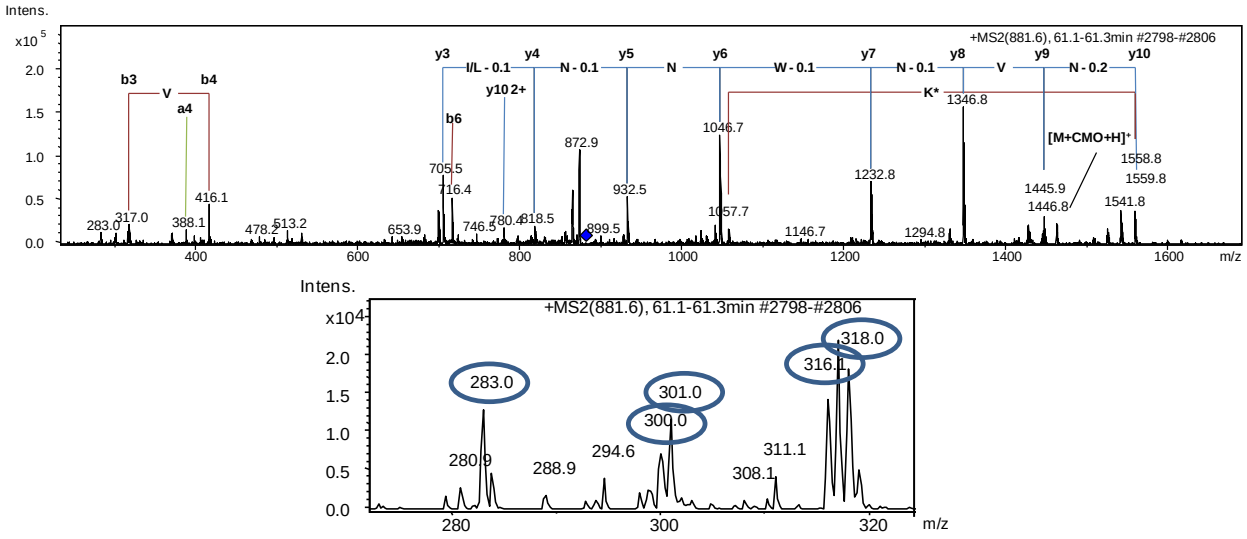
	W	C	T	I	S	S	P	E	E	K	K	Trp	Cys	Thr	Ile	Ser	Ser	Pro	Glu	Glu	Lys	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	W	C	T	I	S	S	P	E	E	K*	K	159.092	319.122	420.170	533.254	620.286	707.318	804.371	933.413	1062.456	1563.704	1691.799
b	W	C	T	I	S	S	P	E	E	K*	K	187.087	347.117	448.165	561.249	648.281	735.313	832.366	961.408	1090.451	1591.698	1719.793
y	W	C	T	I	S	S	P	E	E	K*	K	147.113	648.360	777.403	906.445	1003.498	1090.530	1177.562	1290.646	1391.694	1551.725	1737.804
	11	10	9	8	7	6	5	4	3	2	1	Lys	Lys	Glu	Glu	Pro	Ser	Ser	Ile	Thr	Cys	Trp

Figure 79 MS/MS spectrum of 869.4^{2+} R.WCTISSPEEKK.C 2: Carbamidomethyl (C), 10: ZEN-CMO (K), MW= 1736.8 Da



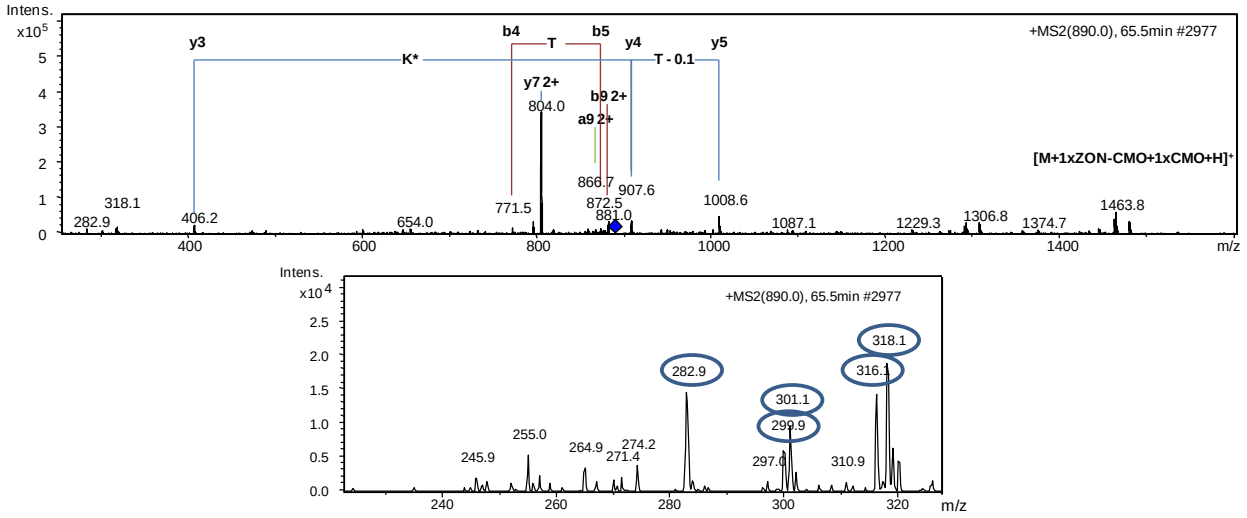
	S	K	F	M	M	F	E	S	Q	N	K	Ser	Lys	Phe	Met	Met	Phe	Glu	Ser	Gln	Asn	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11
a	S	K*	F	M	M	F	E	S	Q	N	K	60.044	561.292	708.360	839.401	970.441	1117.510	1246.552	1333.584	1461.643	1575.686	1703.781
b	S	K*	F	M	M	F	E	S	Q	N	K	88.039	589.287	736.355	867.396	998.436	1145.505	1274.547	1361.579	1489.638	1603.681	1731.776
y	S	K*	F	M	M	F	E	S	Q	N	K	147.113	261.156	389.214	476.246	605.289	752.357	883.398	1014.438	1161.507	1662.754	1749.786
	11	10	9	8	7	6	5	4	3	2	1	Lys	Asn	Gln	Ser	Glu	Phe	Met	Met	Phe	Lys	Ser

Figure 80 MS/MS spectrum of 875.4^{2+} K.SKFMMFESQNK.D 2: ZEN-CMO (K), MW= 1748.8 Da



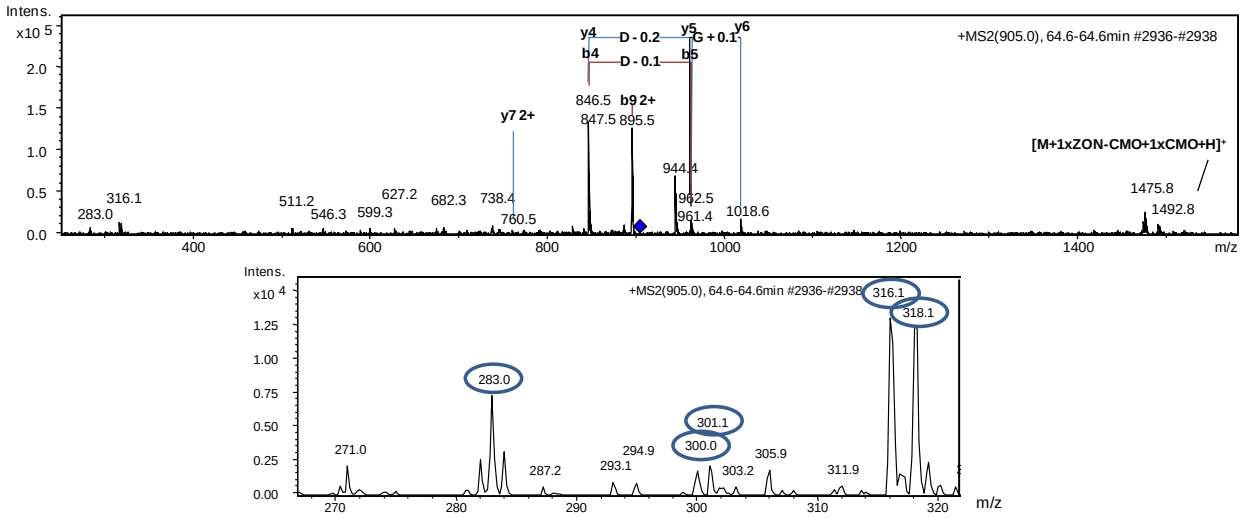
	D	S	N	V	N	W	N	N	L	K	G	K	Asp	Ser	Asn	Val	Asn	Trp	Asn	Asn	Leu	Lys	Gly	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
a	D	S	N	V	N	W	N	N	L	K*	G	K	88.039	175.071	289.114	388.183	502.226	688.305	802.348	916.391	1029.475	1530.722	1587.744	1715.839
b	D	S	N	V	N	W	N	N	L	K*	G	K	116.034	203.066	317.109	416.178	530.221	716.300	830.343	944.386	1057.470	1558.717	1615.739	1743.834
y	D	S	N	V	N	W	N	N	L	K*	G	K	147.113	204.134	705.382	818.466	932.509	1046.552	1232.631	1346.674	1445.742	1559.785	1646.817	1761.844
	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Gly	Lys	Leu	Asn	Asn	Trp	Asn	Val	Asn	Ser	Asp

Figure 81 MS/MS spectrum of 881.4^{2+} K.DSNVNWNNLKGK.K 10: ZEN-CMO (K), MW = 1760.8 Da



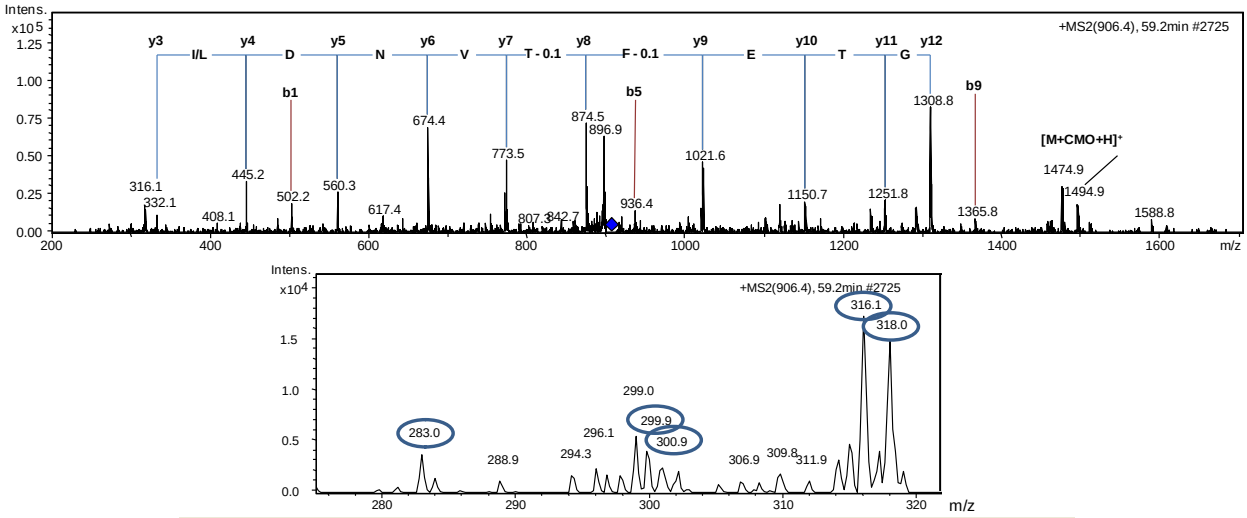
	G	D	P	K	T	K	C	A	R	Gly	Asp	Pro	Lys	Thr	Lys	Cys	Ala	Arg
Ion	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
a	G	D	P	K*	T	K*	C	A	R	30.034	145.061	242.114	743.361	844.409	1345.656	1505.687	1576.724	1732.825
b	G	D	P	K*	T	K*	C	A	R	58.029	173.056	270.108	771.356	872.404	1373.651	1533.682	1604.719	1760.820
y	G	D	P	K*	T	K*	C	A	R	175.119	246.156	406.187	907.434	1008.482	1509.729	1606.782	1721.809	1778.831
	9	8	7	6	5	4	3	2	1	Arg	Ala	Cys	Lys	Thr	Lys	Pro	Asp	Gly

Figure 82 MS/MS spectrum of 889.9^{2+} K.GDPKTKCAR.N 4: ZEN-CMO (K), 6: ZEN-CMO (K), 7: Carbamidomethyl (C), MW= 1777.8 Da



	Q	C	K	G	D	P	K	T	K	Gln	Cys	Lys	Gly	Asp	Pro	Lys	Thr	Lys
Ion	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
a	Q	C	K*	G	D	P	K*	T	K	101.071	261.102	762.349	819.371	934.397	1031.450	1532.698	1633.745	1761.840
b	Q	C	K*	G	D	P	K*	T	K	129.066	289.097	790.344	847.365	962.392	1059.445	1560.693	1661.740	1789.835
y	Q	C	K*	G	D	P	K*	T	K	147.113	248.160	749.408	846.461	961.488	1018.509	1519.757	1679.787	1807.846
	9	8	7	6	5	4	3	2	1	Lys	Thr	Lys	Pro	Asp	Gly	Lys	Cys	Gln

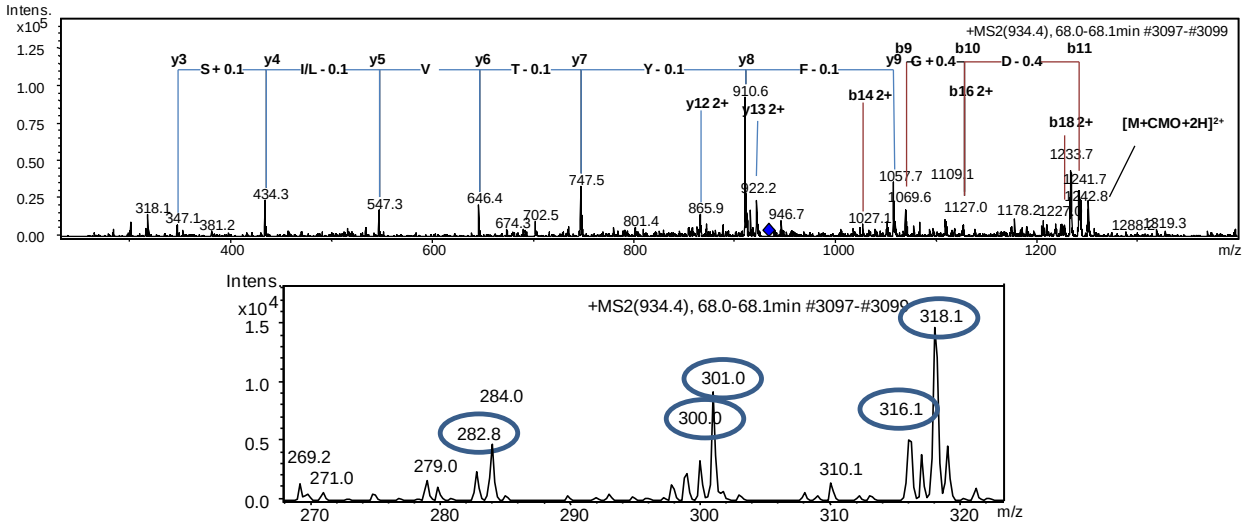
Figure 83 MS/MS spectrum of 904.4^{2+} R.QCKGDPKTK.C 2: Carbamidomethyl (C), 3: ZEN-CMO (K), 7: ZEN-CMO (K), MW = 1806.8 Da



	K	G	T	E	F	T	V	N	D	L	Q	G	K	Lys	Gly	Thr	Glu	Phe	Thr
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6
a	K*	G	T	E	F	T	V	N	D	L	Q	G	K	474.260	531.281	632.329	761.372	908.440	1009.488
b	K*	G	T	E	F	T	V	N	D	L	Q	G	K	502.255	559.276	660.324	789.367	936.435	1037.483
y	K*	G	T	E	F	T	V	N	D	L	Q	G	K	147.113	204.134	332.193	445.277	560.304	674.347
	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Gly	Gln	Leu	Asp	Asn

Val	Asn	Asp	Leu	Gln	Gly	Lys
7	8	9	10	11	12	13
1108.556	1222.599	1337.626	1450.710	1578.769	1635.790	1763.885
1136.551	1250.594	1365.621	1478.705	1606.764	1663.785	1791.880
773.415	874.463	1021.531	1150.574	1251.622	1308.643	1809.891
Val	Thr	Phe	Glu	Thr	Gly	Lys

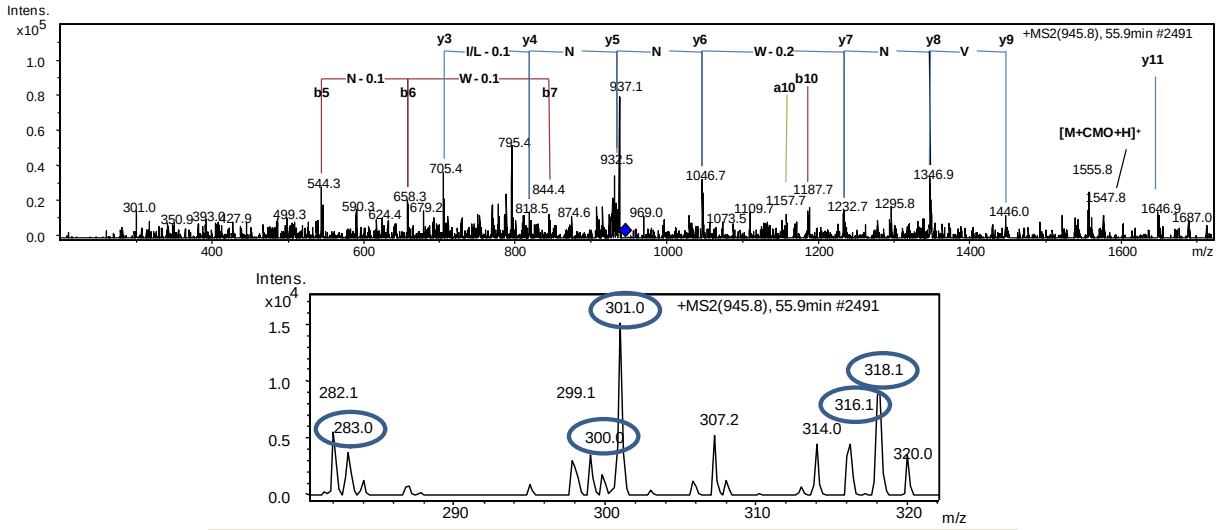
Figure 84 MS/MS spectrum of 905.4^{2+} K.KGTEFTVNDLQGK.T 1: ZEN-CMO (K), MW = 1808.9 Da



	E	G	T	T	Y	K	E	F	L	G	D	K	F	Y	T	V	I	S	S	L	K	Glu	Gly	Thr	Thr	Tyr	Lys	Glu	Phe
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	1	2	3	4	5	6	7	8
a	E	G	T	T	Y	K	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	102.055	159.076	260.124	361.172	524.235	652.330	781.373	928.441
b	E	G	T	T	Y	K	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	130.050	187.071	288.119	389.167	552.230	680.325	809.368	956.436
y	E	G	T	T	Y	K	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	147.113	260.197	347.229	434.261	547.345	646.413	747.461	910.524
	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Leu	Ser	Ser	Ile	Val	Thr	Tyr

Leu	Gly	Asp	Lys	Phe	Tyr	Thr	Val	Ile	Ser	Ser	Leu	Lys
9	10	11	12	13	14	15	16	17	18	19	20	21
1041.525	1098.547	1213.574	1714.821	1861.889	2024.953	2126.000	2225.069	2338.153	2425.185	2512.217	2625.301	2753.396
1069.520	1126.542	1241.568	1742.816	1889.884	2052.948	2153.995	2253.064	2366.148	2453.180	2540.212	2653.296	2781.391
1057.593	1558.840	1673.867	1730.889	1843.973	1991.041	2120.084	2248.179	2411.242	2512.290	2613.337	2670.359	2799.402
Phe	Lys	Asp	Gly	Leu	Phe	Glu	Lys	Tyr	Thr	Thr	Gly	Glu

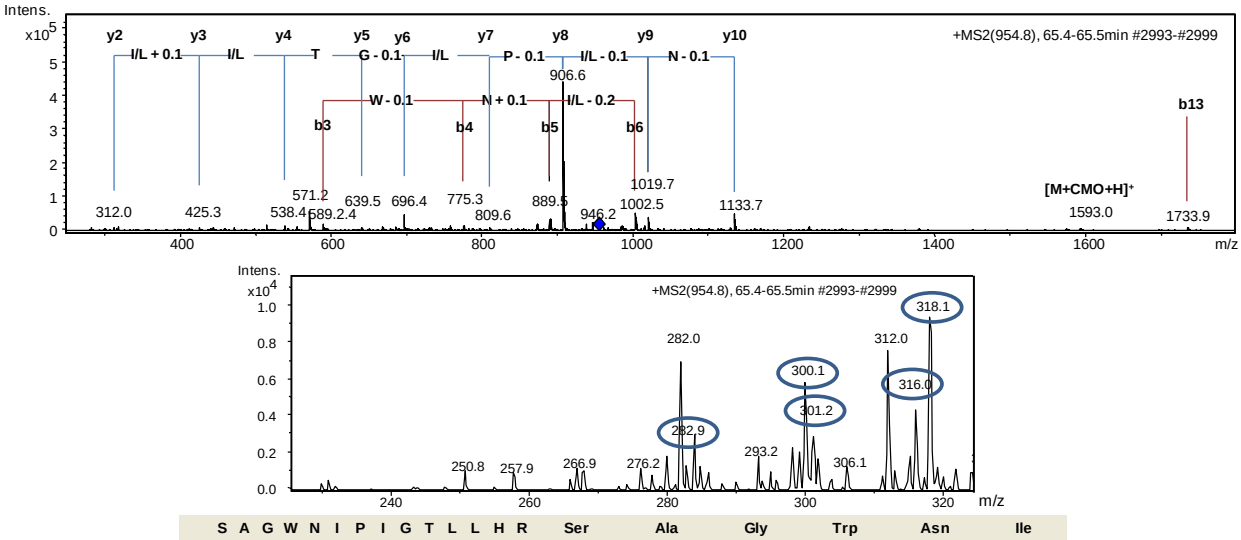
Figure 85 MS/MS spectrum of 933.8^{3+} R.EGTTYKEFLGDKFYTVISSLK.T 12: ZEN-CMO (K), MW= 2798.4 Da



	K	D	S	N	V	N	W	N	N	L	K	G	K	Lys	Asp	Ser	Asn	Val
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5
a	K	D	S	N	V	N	W	N	N	L	K*	G	K	101.107	216.134	303.166	417.209	516.278
b	K	D	S	N	V	N	W	N	N	L	K*	G	K	129.102	244.129	331.161	445.204	544.273
y	K	D	S	N	V	N	W	N	N	L	K*	G	K	147.113	204.134	705.382	818.466	932.509
	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Gly	Lys	Leu	Asn

	Asn	Trp	Asn	Asn	Leu	Lys	Gly	Lys
	6	7	8	9	10	11	12	13
b	630.321	816.400	930.443	1044.486	1157.570	1658.817	1715.839	1843.934
b	658.315	844.395	958.438	1072.481	1185.565	1686.812	1743.834	1871.929
y	1046.552	1232.631	1346.674	1445.742	1559.785	1646.817	1761.844	1889.939
	Asn	Trp	Asn	Val	Asn	Ser	Asp	Lys

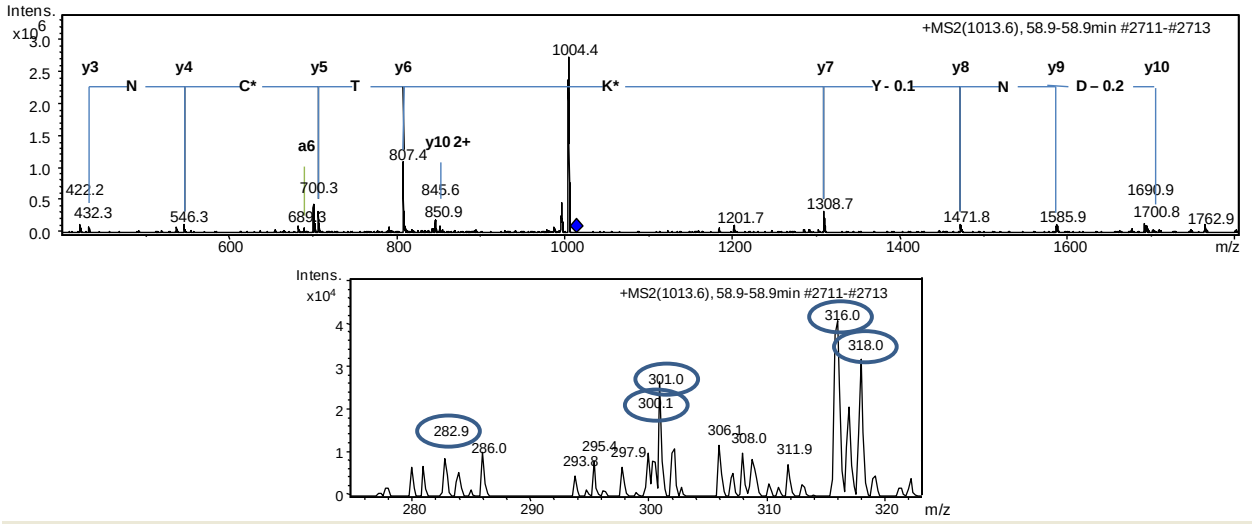
Figure 86 MS/MS spectrum of 945.4^{2+} R.KDSNVNWNNLKGK.K 11: ZEN-CMO (K), MW=1888.9 Da



	S	A	G	W	N	I	P	I	G	T	L	L	H	R	Ser	Ala	Gly	Trp	Asn	Ile
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6
a	S*	A	G	W	N	I	P	I	G	T	L	L	H	R	433.197	504.234	561.256	747.335	861.378	974.462
b	S*	A	G	W	N	I	P	I	G	T	L	L	H	R	461.192	532.229	589.250	775.330	889.373	1002.457
y	S*	A	G	W	N	I	P	I	G	T	L	L	H	R	175.119	312.178	425.262	538.346	639.394	696.415
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Arg	His	Leu	Leu	Thr	Gly

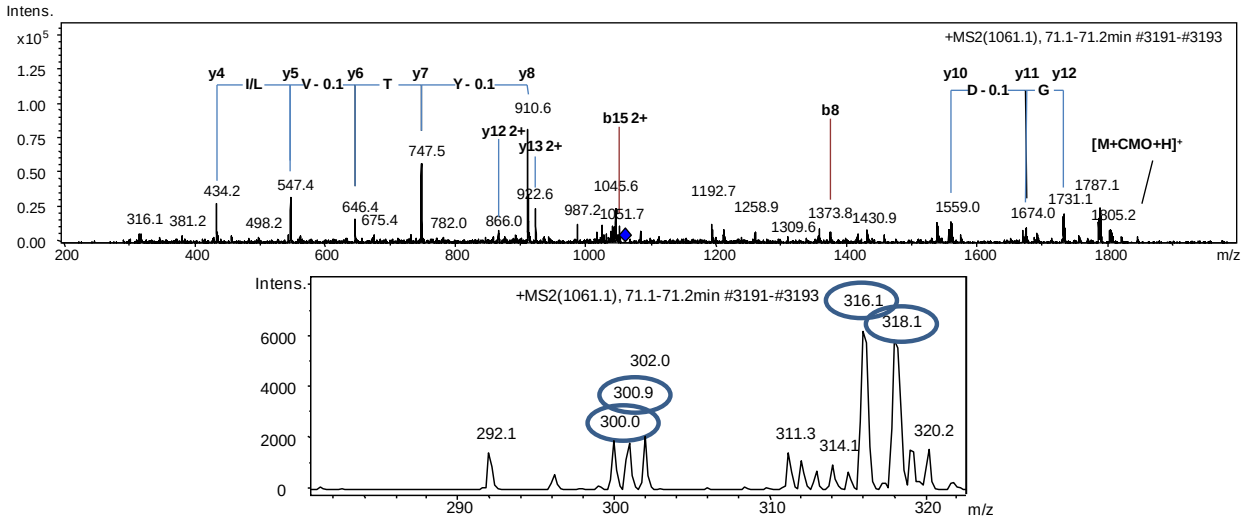
	Ile	Pro	Ile	Gly	Thr	Leu	Leu	His	Arg
	6	7	8	9	10	11	12	13	14
b	974.462	1071.515	1184.599	1241.620	1342.668	1455.752	1568.836	1705.895	1861.996
b	1002.457	1099.509	1212.594	1269.615	1370.663	1483.747	1596.831	1733.890	1889.991
y	696.415	809.499	906.552	1019.636	1133.679	1319.758	1376.780	1447.817	1908.001
	Gly	Ile	Pro	Ile	Asn	Trp	Gly	Ala	Ser

Figure 87 MS/MS spectrum of 954.5^{2+} R.SAGWNIPIGTLLHR.G 1: ZEN-CMO (S), MW = 1907.0 Da



	Q	P	V	D	N	Y	K	T	C	N	W	A	R	Gln	Pro	Val	Asp	Asn	Tyr	Lys	Thr	Cys	Asn	Trp	Ala	Arg
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9	10	11	12	13
a	Q	P	V	D	N	Y	K*	T	C	N	W	A	R	101.071	198.124	297.192	412.219	526.262	689.325	1190.573	1291.620	1451.651	1565.694	1751.773	1822.810	1978.912
b	Q	P	V	D	N	Y	K*	T	C	N	W	A	R	129.066	226.119	325.187	440.214	554.257	717.320	1218.568	1319.615	1479.646	1593.689	1779.768	1850.805	2006.907
y	Q	P	V	D	N	Y	K*	T	C	N	W	A	R	175.119	246.156	432.235	546.278	706.309	807.357	1308.604	1471.667	1585.710	1700.737	1799.806	1896.859	2024.917
	13	12	11	10	9	8	7	6	5	4	3	2	1	Arg	Ala	Trp	Asn	Cys	Thr	Lys	Tyr	Asn	Asp	Val	Pro	Gln

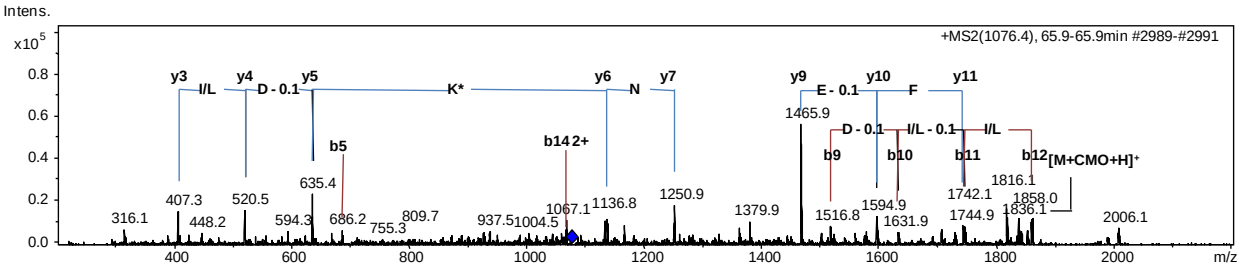
Figure 88 MS/MS spectrum of 1013.0^{2+} R.QPVDNYKTCNWAR.V 7: ZEN-CMO (K), 9: Carbamidomethyl (C), MW = 2023.9 Da

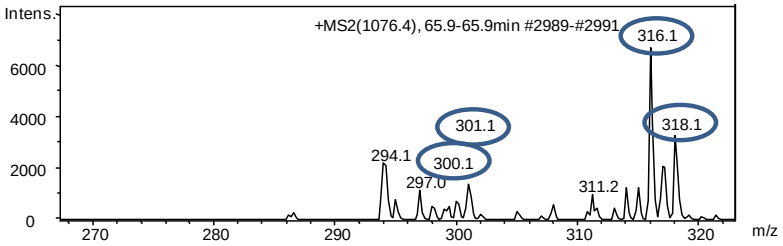


	E	F	L	G	D	K	F	Y	T	V	I	S	S	L	K	Glu	Phe	Leu	Gly	Asp	Lys
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1	2	3	4	5	6
a	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	102.055	249.123	362.207	419.229	534.256	1035.503
b	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	130.050	277.118	390.202	447.224	562.251	1063.498
y	E	F	L	G	D	K*	F	Y	T	V	I	S	S	L	K	147.113	260.197	347.229	434.261	547.345	646.413
	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Leu	Ser	Ser	Ile	Val

Phe	Tyr	Thr	Val	Ile	Ser	Ser	Leu	Lys
7	8	9	10	11	12	13	14	15
1182.572	1345.635	1446.683	1545.751	1658.835	1745.867	1832.899	1945.983	2074.078
1210.567	1373.630	1474.678	1573.746	1686.830	1773.862	1860.894	1973.978	2102.073
747.461	910.524	1057.593	1558.840	1673.867	1730.889	1843.973	1991.041	2120.084
Thr	Tyr	Phe	Lys	Asp	Gly	Leu	Phe	Glu

Figure 89 MS/MS spectrum of 1060.5^{2+} K.EFLGDKFYTVISSLK.T 6: ZEN-CMO (K), MW = 2119.1 Da





	F	M	M	F	E	S	Q	N	K	D	L	L	F	K	Phe	Met	Met	Phe	Glu	Ser
Ion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	4	5	6
a	F	M	M	F	E	S	Q	N	K*	D	L	L	F	K	120.081	251.121	382.162	529.230	658.273	745.305
b	F	M	M	F	E	S	Q	N	K*	D	L	L	F	K	148.076	279.116	410.157	557.225	686.268	773.300
y	F	M	M	F	E	S	Q	N	K*	D	L	L	F	K	147.113	294.181	407.265	520.349	635.376	1136.624
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Lys	Phe	Leu	Leu	Asp	Lys

Gln	Asn	Lys	Asp	Leu	Leu	Phe	Lys
7	8	9	10	11	12	13	14
873.363	987.406	1488.654	1603.681	1716.765	1829.849	1976.917	2105.012
901.358	1015.401	1516.649	1631.676	1744.760	1857.844	2004.912	2133.007
1250.667	1378.725	1465.757	1594.800	1741.868	1872.909	2003.949	2151.018
Asn	Gln	Ser	Glu	Phe	Met	Met	Phe

Figure 90 MS/MS spectrum of 1076.0^{2+} K.FMMFESQNKDLLFK.D 9: ZEN-CMO (K), MW = 2150.0 Da