# UNIVERSITA KARLOVA V PRAZE

# FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra organické a anorganické chemie


## Analysis of farnesylated peptides and proteins using LC-MS

Masters thesis


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# Contents

# Introduction

Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify the compounds and to elucidate the structure and chemical properties of molecules. Research, which was done in past few decades made mass spectrometry a highly sensitive method that is capable of analyzing very minute quantities of compounds with a precision within 0.01 %, all can be done in chemically complex mixtures. The development of the instrument, such as implementation of new types of analyzers, e.g. triple-quadrupole or ion trap, introduction of new ways of ionization, e.g. electrospray and matrix-assisted laser desorption ionization, made this method highly popular also in biochemical research. All this as well as increased commercial availability of the instrument helped to extend its use from simple molecular weight characterization. Today mass spectrometry is utilized in various branches of industry and research.

In biotechnology it is used to analyze oligonucleotides, peptides and proteins. It is possible to obtain their sequence, both identify them de novo or confirm their sequence with the help of databases. Also post-translational modifications, number of disulphide bridges, macromolecular structure determination and enzyme formation and reactions can be studied by mass spectrometry. In pharmaceutical branch the use is in drug discovery, combinatorial chemistry, pharmacokinetics and determining of purity. Clinical use can be hemoglobin analysis and various drug testing, e.g. monitoring of breath of patients during surgery by anesthesiologist. Environmental application represents testing can be used to detect pollution of nature by products of industry. Last but not least mass spectrometry is used to study food contamination, e.g. detect dioxins in fish or determine whether honey is adulterated with syrup. This method is also used in such a different field as astrophysics, because it determines the composition of molecular species found in space.

# Objective of the thesis

Farnesylation is a sort of posttranslational modification of proteins called the protein prenylation. It presents the addition of a lipophilic isoprenoid moiety with fifteen carbons to the virgin protein. This modification enables the proteins to be anchored via the new farnesyl moiety in the plasmatic membrane of the cell. There the prenylated proteins usually serve as transducers of signals (e.g. RAS proteins). If there is a problem with the prenylated protein, for example as a result of mutation, it can lead to serious diseases, such as cancer. The reason for this is uncontrollable signal transduction and the consequent cell proliferation.

There are several drug groups, which affect the prenylated protein. In order to monitor the effect of these drugs on levels of prenylated proteins in cells or to easily diagnose a cancer resulted from over expression of these proteins, a method has to be developed to screen them. Electrospray mass spectrometry (ESI/MS) coupled with liquid chromatography was chosen as a method to analyze farnesylated proteins. This method is popular in protein analysis, accurate and relatively easily available.

The objective of this work should be the analysis of farnesylated proteins by MS, characterize the nature of their cleavages and obtain detailed information about the resulting MS spectra. To do so, simple peptides will be analyzed at first and then with the knowledge obtained from their spectra, complex samples from cells will be searched for farnesylated proteins.

# Theoretical part

# Fundamentals of mass spectrometry

Mass spectrometry (MS) is an analytical technique that identifies the chemical composition of a compound or sample on the basis of the mass-to-charge ratio of charged particles. It works by generating charged molecules or molecular fragments either in a high vacuum or immediately prior to the sample entering the high vacuum region. The ionized molecules have to be generated in the gas phase, thus it is relatively simple to connect gas chromatography as a separative method with analysis from mass spectrometer, but it took more time until an interface was developed to produce charged molecules between liquid chromatography and mass spectrometer. Today the most common interfaces are the electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), both are used in protein analysis. Once the molecules are charged and in the gas phase, they can be manipulated by the application of either electric or magnetic fields to enable the determination of their molecular weight and the molecular weight of any fragments which are produced by the molecule breaking up[1].

## Instrumentation

The design of each mass spectrometer has three main parts: an **ion source**, which transforms the molecules in a sample into ionized fragments (electrospray ionization was used in the further experiments); a **mass analyzer**, which sorts the ions by their masses by applying electric and magnetic fields (the device used had a linear ion trap as an analyzer); and a **detector**, which measures the value of some indicator quantity and thus provides data for calculating the abundances each ion fragment present.

### Ion source

Electrospray is a method by which ions, present in solution, can be transferred to the gas phase. Many samples that previously were not suitable for mass analysis (for example, heat-labile compounds or high molecular

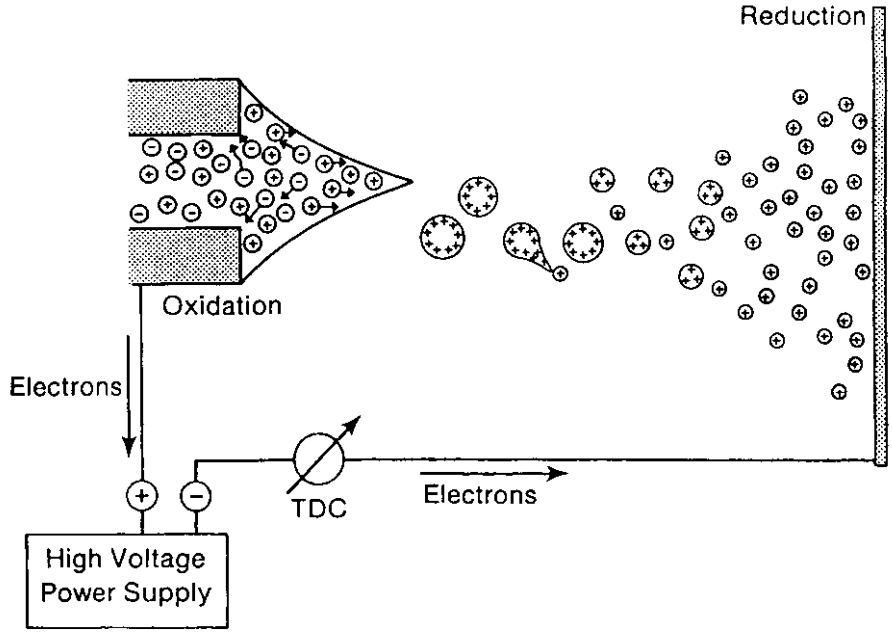
weight compounds) can be analyzed by the use of ESI. ESI can be used to analyze any polar compound that makes a preformed ion in solution. Thus some compounds can be analyzed with some suitable additive, forming electrolyte. The ESI enables the interfacing of LC-MS with a relatively high flow rates – up to 1 mL/min, but best works at 150 – 200 μL/min flow rate. When analyzing peptides however, flow rates of couple μL/min produce the best results, because as a concentration dependent device, ESI maximizes the concentration of the peptide that is subjected to the analysis (Yates 1998).

An electric field is applied to the tip of a capillary, which is containing the analyzed solution (leading e.g. from a column of HPLC). The presence of the field leads to formation of a dipolar layer at the meniscus of the liquid at the capillary tip. When the capillary is positive, positive electrolyte ions from the double layer are near the surface and destabilize the meniscus. A cone and a liquid jet forms and is emitting a fine mist of positively charged droplets, these are traveling in a potential and pressure gradient towards the analyzer (see Fig I). Sometimes an uncharged carrier gas such as nitrogen is sometimes used to help nebulize the liquid. The capillary removes the negative ions from the solution. Solvent evaporation from the charged droplets is accelerated by flow of nitrogen bath gas, placed before the skimmer device, which is in the entrance to the analyzer. The droplet shrinkage leads to increased coulombic repulsion and the release of coulombic strain by droplet fission. Repeated evaporation and fission of parent and offspring droplets lead to formation of very small droplets (10 nm diameter). Each droplet carries approximately 2 % of the parent mass, but 15 % of the parent charge. The whole procedure takes about 500 μs which is close to the resident time of droplets before intake into the mass spectrometric sampling system – the ion sweep cone and skimmer[2].

ESI is the softest ionization technique – it does not cleave the molecules, which are analyzed. Instead it produces multiply charged ions – $[M + nH]^{n+}$ ($[M - nH]^{n-}$ respectively when the capillary is negatively charged and reduction occurs there) - this makes it possible to analyze molecule with a practical mass up to 100 kDa, which without being multiply charged would

be outwith the separation range of most instruments. The range practically extends only to 3 kDa for single-charged ions. On the other hand, multiple charging is sometimes confusing.

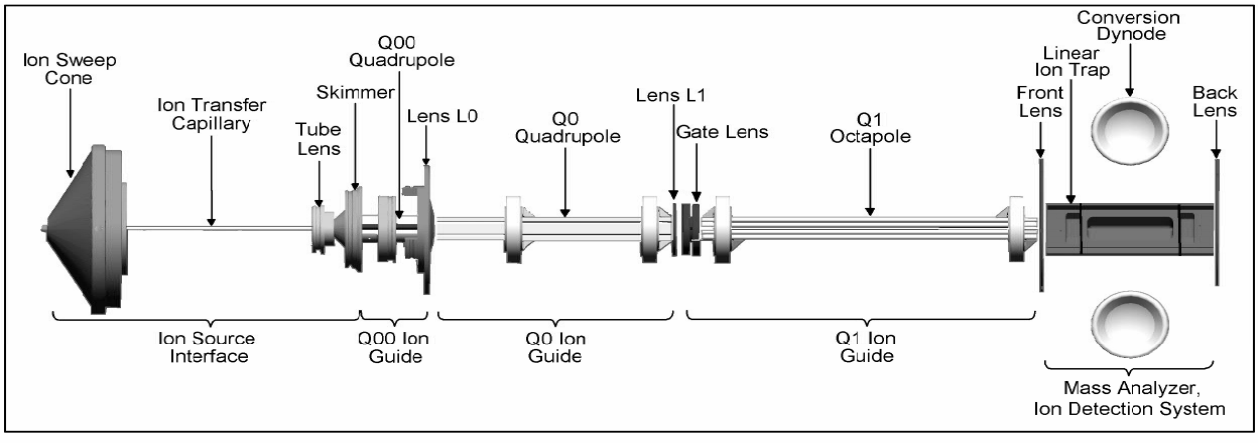
**Fig. I: Basic principle of electrospray ionization**




The produced ions are now in an ion source interface of the machine, this is a place which assists in final desolvation of the ions by high temperature and the ions continue further to the high vacuum resort of the machine. The skimmer acts as a vacuum baffle between the higher pressure ion source interface region (at 1 Torr) and the lower pressure ion guide region (at 50 mTorr) of the vacuum manifold.

The ions have now entered the ion optics region, which is nothing more than a device to focus the ion transmission to the analyzer. It uses electric field, which focuses the beam of ions. It also accelerates or decelerates the ions as they pass through the region. It also further magnifies the vacuum until approximately 1 mTorr.

**Fig. II: Scheme of the evacuated components of a mass spectrometer**



**Mass analyzer and detector**

The mass analyzer is the site of mass analysis. The front and back lenses are disks with a circular hole in the center through which the ion beam can pass. The purpose of the front and back lenses is to provide conductance limits. The linear ion trap is a square array of three sets of hyperbolic rods. The middle part of the trap is provided with holes to radial exit the ions for detection. The three pair of quadrupole rods use each different sets of high voltage with variable amplitude (0-10 kV) to center the ions in the central part of the ion trap but with a constant frequency about 1 MHz.

When the amplitude of the main voltage is low, all ions above a minimum mass-to-charge ratio are trapped. This voltage is referred to as the storage voltage. As the main voltage increases, ions of increasing mass-to-charge ratio become successively unstable in the radial direction, gaining kinetic energy and are ejected from the mass analyzer. The voltage at which an ion is ejected from the mass analyzer is defined as its resonance voltage. The ejection of ions of each mass-to-charge ratio occurs over a very short time. These ions are detected by the ion detection system. The ion isolation waveform voltage consists of a distribution of frequencies containing all resonance frequencies except for those corresponding to the ions to be trapped. It ejects all ions except those of a selected mass-to-charge ratio or narrow ranges of mass-to-charge ratios. The mass analyzer cavity contains helium that is used as a damping gas and a collision activation partner. The helium damping gas enters the mass analyzer cavity through a gap between the quadrupole rods. The collisions of the ions entering the mass analyzer with the helium slow the ions so that they can be trapped by the electro-magnetic field in the mass analyzer. The presence of helium in the mass analyzer cavity

significantly enhances sensitivity and mass spectral resolution. Before the ejection from the mass analyzer cavity, sample ions collide with helium atoms. These collisions reduce the kinetic energy of the ions, thereby damping the amplitude of their oscillations. As a result, the ions are focused to the axis of the cavity rather than being allowed to spread throughout the cavity.

After many collisions with the helium damping gas, which is present in the mass analyzer at the voltage just below the resonance voltage, the ion is gaining internal energy. After gaining sufficient internal energy from the resulting collisions, the parent ion dissociates into one or more product ions. The product ions are then mass analyzed and as a MS/MS spectrum it provides valuable information about the structure. This can of course happen many times even from the offspring ions thus creating $MS^n$ spectra.

The determination of the mass of an ion from its mass-to-charge ratio may be complicated by the fact that the charge state of the ion may be unknown due to multiple charging by ESI. We can therefore use a zoom scan. It is a high resolution MS scan that allows us to determine the charge state and molecular weight of an ion. If the isotopic peaks are 1 amu apart, the ion has a charge state of ±1. If the isotopic peaks are 0.5 amu apart, the ion has a charge state of ±2. If the isotopic peaks are 0.33 amu apart, the ion has a charge state of ±3, and so on. We can then determine the molecular weight of the ion from knowledge of the charge state and mass-to-charge ratio of the ion.

The ion **detector**s are using conversion dynodes, which generate a measurable electric current after the ion strikes the surface of the dynode.

## Analysis of peptides and proteins

Electrospray ionization mass spectrometry is an important tool for the analysis of peptides and proteins. By this method a simple molecular weight determination of an intact protein can be done, in order to quickly check it or further analyze it by additional fragmentation in the device (MS/MS). The protein can be also quite easily sequenced, confirming the identity of the

protein and thus discovering the primary structure of the protein. This approach is called as top-down strategy of protein analysis. Different approach is a bottom-up strategy, in which first a protease (such as trypsin) is used to digest the protein to smaller peptides and these are further analyzed. From the MS/MS data a so called peptide mass fingerprint is obtained, characterizing the peptides. This can be matched with existing protein databases containing peptide mass maps and in such way identify the protein. Also three dimensional structure of the protein can be determined by several methods using mass spectrometry as well.

To determine the sequence of the protein, the MS/MS spectra are used as was already written before, this sequencing is called the tandem mass spectrometry. It is based on a parent (precursor) ion fragmentation leading to a daughter ion fragment (MS/MS spectrum), which can be further broken down by the same mechanism giving us the granddaughter ion (an MS/MS/MS spectrum = $MS^3$). Most effective in peptide mapping is the combination of proteolytic digestion with MS/MS. Tandem spectra derived from trypsin digestion is very useful because basic residues on the C-terminus (Arg and Lys) and the N-terminus (if unblocked) yield a double-charged precursor ion (see Fig. IV). Dissociation of this molecule produces an easily interpretable spectrum with single-charged fragments. Identification of an unknown protein can rapidly proceed from the masses of its proteolytic peptides. There are several computer- based searching routines in which the measured peptide masses along with the MS/MS data are entered into the program and compared to theoretical proteolytic masses of proteins in a given sequence database[2].

The nomenclature to identify the peptide cleavage products can be described in short, fragment ions with the charge residing on the N-terminus of the peptide are denoted as $a_n$, $b_n$ and $c_n$; product ions and fragments with the charge residing on the C-terminus are identified as $x_n$, $y_n$ and $z_n$ ions, where n designated the residue number (counting from the N- or C- terminus respectively).

**Fig. III: Types of fragments observed in MS/MS spectrum (Johnson 1987)**

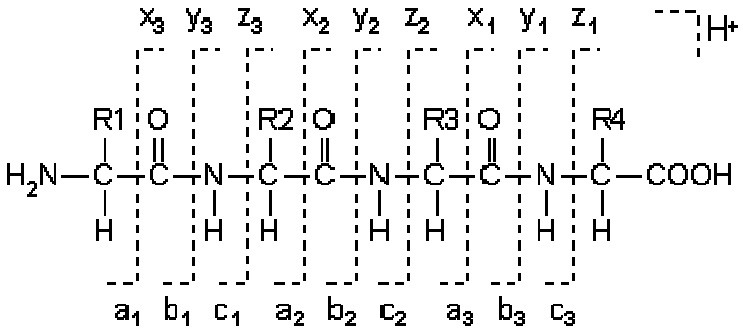
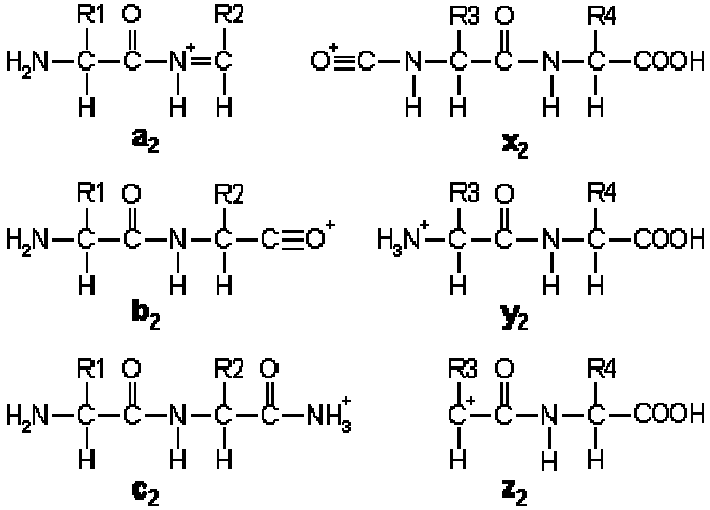



**Fig. IV: Formation of an additional charge after the protein cleavage**




In lower energy collisions, which are induced by the ion trap, peptide carrying a positive charge fragments mainly along its backbone, generating predominantly b and y ions. In addition, peaks are seen for ions which have lost ammonia (-17 Da) and water (-18 Da). Also internal fragments are produced, usually by a simultaneous b and y cleavage, further complicating the spectrum.

With the information gained from the MS/MS spectra the peptide, these can be compared against a database in order to identify the protein or at least relate it closely to a known protein. There is a considerable amount of databases, which collect the MS information about proteins from different species. These databases are downloaded to linking programs (e.g. Bioworks browser), which are using a cross-correlation function – sequest. This method

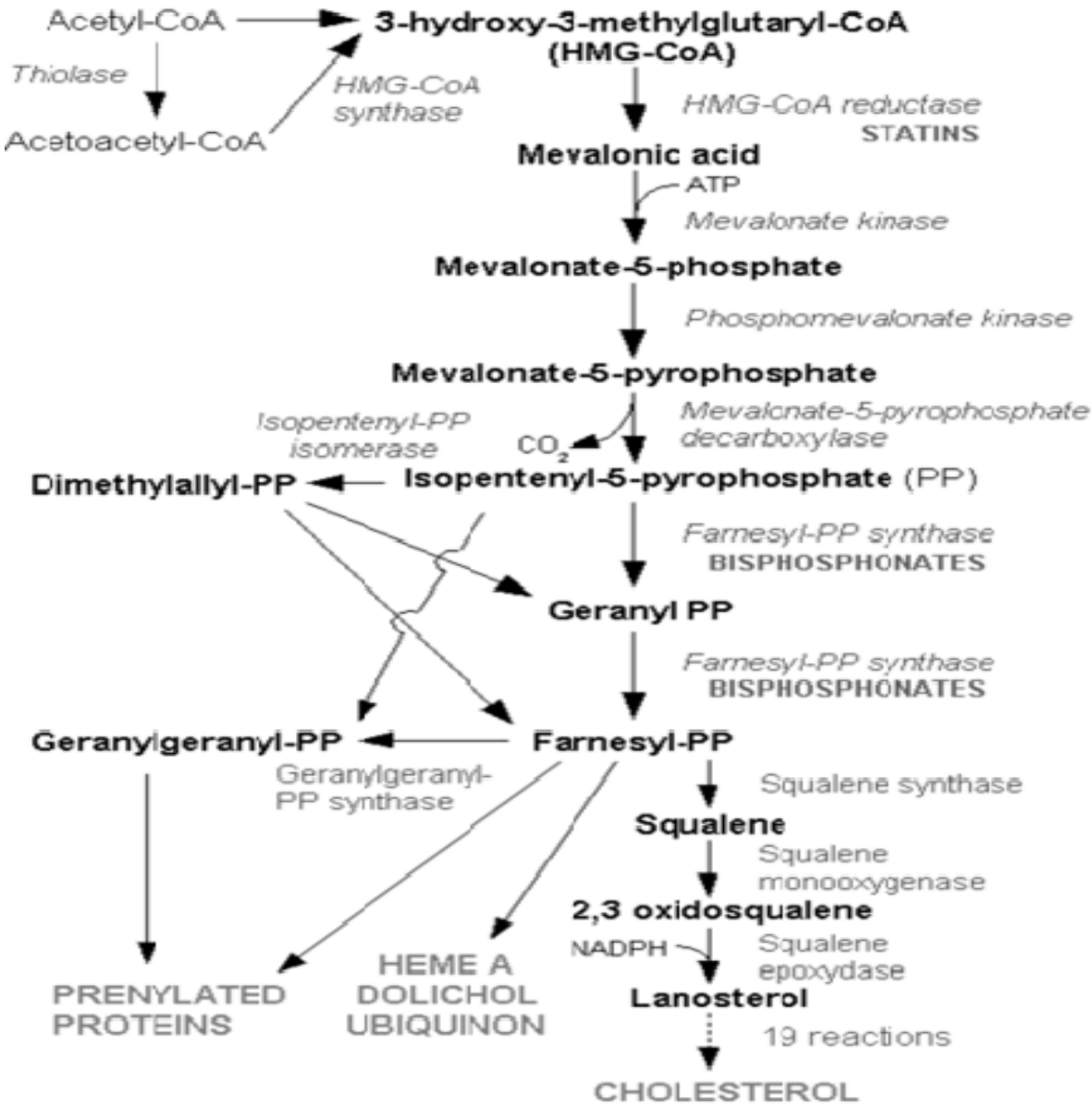
determines the similarity of the spectrum obtained experimentally and the spectrum information from the database. Usually, when analyzing a protein, two or more identical peptide fragment sequences are enough to prove the protein in the sample. Proteins are of course rarely without a post translational modification that changes the protein mass from simple sum of amino acid mass. These include e.g. phosporylation, glycation and also prenylation, which is the subject of this work. Therefore, when analyzing proteins, it is necessary to consider this and eventually adjust the search conditions of the sequest program[1, 3, 4].

## Protein prenylation

The prenylation (isoprenylation) of proteins is one of the most recently discovered modifications of eukaryotic cell proteins. Some prenylated proteins are initially produced with a C-terminal sequence motif CaaX (C is cysteine, a is usually an aliphatic residue and X is typically serine, methionine or glutamine). Prenylation occurs by enzyme catalyzed attachment of a 15-carbon isoprenoid farnesyl group to cysteine via a thioether linkage. After the prenylation, aaX is released by a membrane bound endoprotease and the newly exposed S-farnesylcysteine is methylated on its carboxyl group by a membrane bound methyltransferase. In other type of prenylated proteins, the attachment is not a farnesyl group, but a 20-carbon isoprenoid geranylgeranyl, while the methylation occurs as for the farnesylated proteins. The attachment of prenylgroups is catalyzed by distinct protein transferases. The production of farnesyl is a part of the mevalonate pathway of cholesterol biosynthesis, while geranylgeranyl derives from farnesyl[5] (see Fig. V).

The role of protein prenylation, this insertion of a lipophilic side chain to a molecule of polar protein, is mainly in anchoring of the protein to the lipid bilayers of plasmatic membrane in eukaryotic cell. They also contribute to signal transduction not only by the insertion of the receptor proteins to the cell membrane, but by a regulated vesicle transport and specific protein-protein interaction[6].

**Fig. V: Biosynthesis of isoprenoids for protein prenylation and places of drug intervention (Wiki)**




Some examples of prenylated proteins are e.g. rhodopsin kinase, subunit of cGMP phosphodiesterase, subsets of small GTP binding proteins and many subunits of different heterotrimeric G proteins. Perhaps the most studied prenylated proteins are the RAS superfamily of small GTPases. These proteins function as regulators of important biological processes including transmembrane signal transduction, cytoskeletal reorganisation, gene expression, intracellular vesicle trafficking, microtubule organization and nucleocytoplasmic transport. They all possess the ability to be GTB-bound and therefore biologically active and biologically inactive, while GDP-bound.

Each cycle of activation and inactivation is coupled with transduction of an upstream signal to downstream effectors. The role of the RAS superfamily of GTPases in carcinogenesis is well established; approximately 20 % of human cancers are caused by a mutational activation of RAS. All these mutations stabilize RAS in the constitutively active GTP-bound conformation, leading to overexpression, mutational activation or amplification at certain growth factor receptors, thus leading to cancer[7].

The process of protein prenylation can be affected pharmacologically by several types of drugs. They can be distinguished on inhibitors of mevalonate pathways and prenylation inhibitors. Currently there are also drugs in development to affect the post-prenylated state of the proteins.

Several enzymes in the mevalonate pathway have been targeted for anticancer drug develelopmet. The statins are normally used to reduce the production of cholesterol, thus reducing the risk of cardiovascular diseases. By inhibiting the enzyme HMG-CoA reductase it also inhibits the formation of downstream isoprenoids farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate, which are used as substrates for prenylation (see Fig. V). The results of anticancer activity of statins are only modest. Different enzymes in the mevalonate pathways are inhibited by bisphosphonates. These inhibit isopentenylpyrophophate isomerase and FPP synthase. Bisphosphonates again have only limited anticancer effects, but they are widely used in therapy of osteoporosis, because they inhibit farnesylation of RAS signaling proteins in osteoclasts. This inhibits their proper function and results in a bigger bone density.

The prenylation inhibitors or farnesyltranferase inhibitors are competing with FPP for binding to farnesyltransferase. This class of drugs includes for example tipifarnib and lonafarnib. The antitumour activity of these drugs as single agents is not very high[7].

Prenylated proteins and the process of their formation are promising targets of new drug discoveries, curing various diseases in which these proteins are involved, mainly some forms of cancer. A reliable and simple screening method of these proteins should therefore be developed to

determine their presence and level in cells and so evaluating the drug effects.

# Methods

Primarily it was crucial to obtain an idea, how the spectra of farnesylated peptides look like and how do they differ from a virgin peptide spectra. The nature of their cleavages will afterwards aid us in identifying the farnesylated proteins in much more difficult sample made from cells. Basic understanding of the fragmentation sites and appearance of farnesyl, when broken from the molecule of the peptide should be deduced from the spectra. For this, two peptides were chosen – CVLS and a longer K-peptide. Afterwards the cleavages will also be checked on farnesylated bovine albumin that should imitate the farnesylated proteins more than just simple peptides. Bovine albumin will also serve as an object to study the separation of farnesylated and non-farnesylated proteins prior to chromatographic separation. Finally, it will be tried to find farnesylated proteins in a sample made of MCF 7 breast cancer cells. This sample should contain only membrane proteins, as it is expected that this fraction should contain the proteins of interest – farnesylated ones.

## CVLS

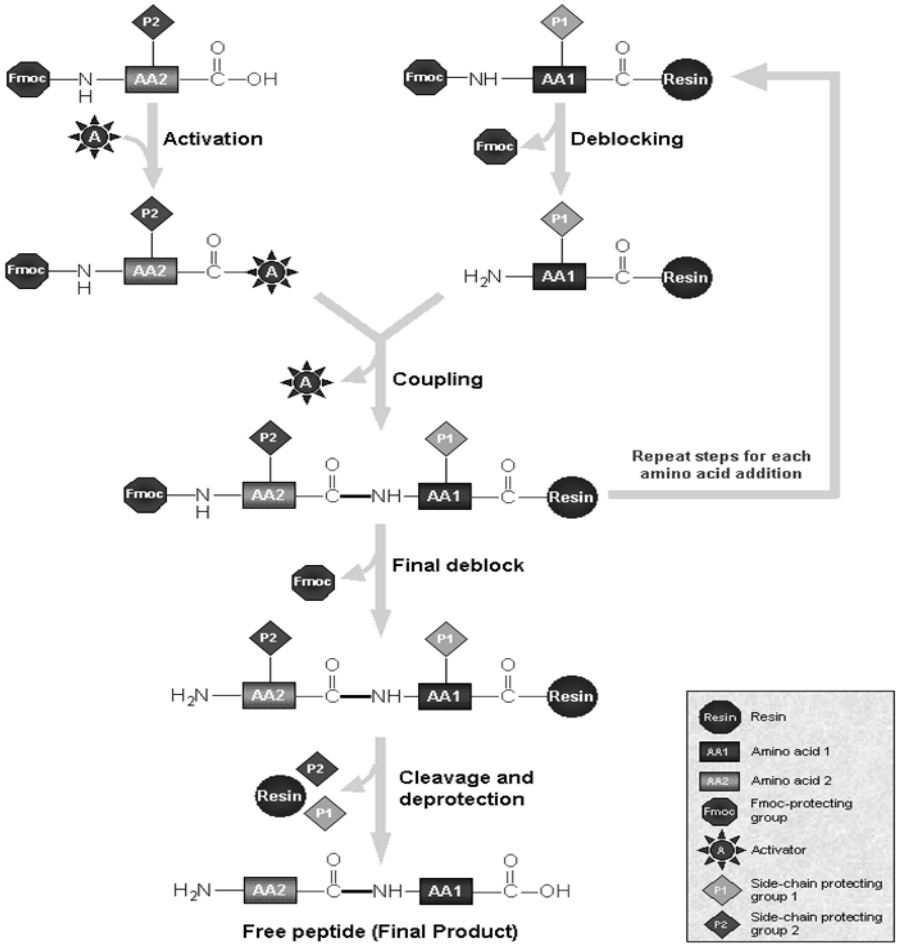
First a simple peptide was chosen to see the difference between the peptide itself and afterwards with the farnesyl moiety attached, when analyzed by MS. The peptide CVLS was chosen because of its similarity with the ending sequence of many prenylated proteins – CaaX, e.g. Ras proteins or subunits of G proteins and so on.

### Peptide synthesis

The peptide was obtained by standard manual Fmoc solid phase synthesis of peptides. The solid phase was represented in this case by serine resin. Serine was linked to the functionalized polystyrene/polyethylene glycol by an ester bond, which is TFA labile. The serine hydroxyl was protected by a tert-butyl group, while the amino group was protected by Fmoc (flurenylmethoxycarbonyl). To react with another building block in the synthesis, the Fmoc group had to be removed, this was done by piperidine. Now, the serine was prepared to react with another amino acid – Fmoc

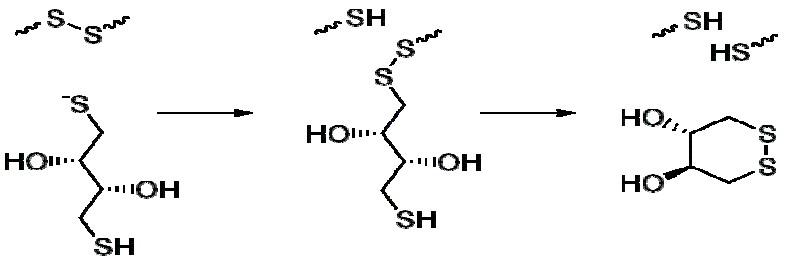
leucine. Fmoc again was protecting the amino group and the amino acid was activated by DIPEA and HBTU. Then valine and cysteine were added in the same way, so that the wanted peptide was synthesized. All that was left to do, was to cleave the peptide from the resin and rest of the protective groups, this was achieved by incubation with TFA.

**Fig. VI: Solid phase peptide synthesis**

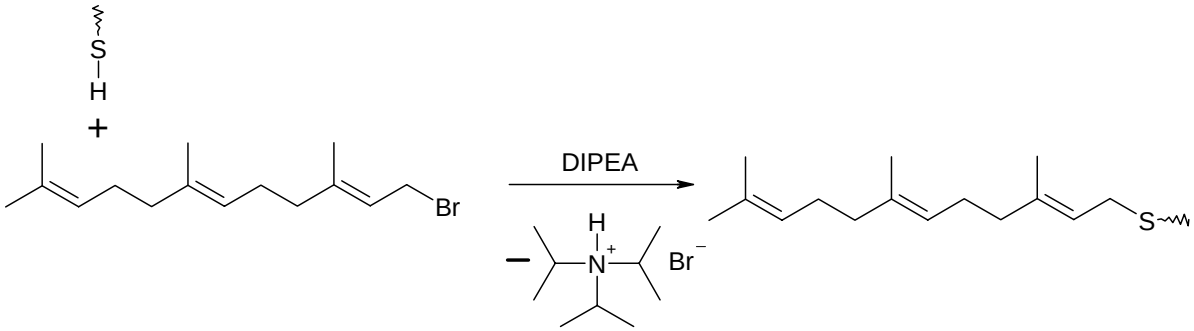



## Farnesylation

Because the CVLS peptide formed a dimer rather than staying in a form with free SH group, the peptide had to be reduced. This was done by incubating it with DTT.



Then the peptide was subjected to react with DIPEA and farnesyl bromide. This reaction is a modified procedure according to Appels[8, 9].




The farnesylated CVLS and the native CVLS were analyzed and their structures were confirmed. However the exact state in which is the farnesyl moiety, when leaving the molecule, could not be pointed out. It was decided to view the farnesyl and its behavior on a different molecule. A molecule, that is closer to the proteins, which this method should be developed for. Albumin was chosen as one of the most common (and cheapest) proteins.
But first, also the so-called K-peptide was farnesylated and analyzed.

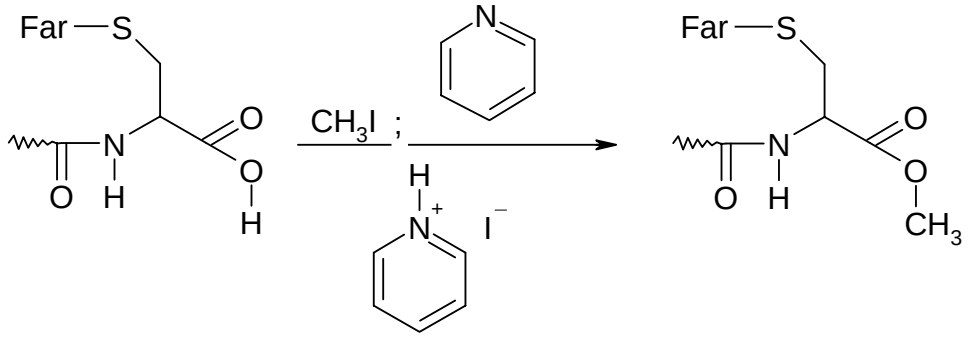
## K-peptide

The K-peptide was chosen because it has cysteine on its carboxyl end, perfect for simulation of most prenylated proteins in organism, where this cysteine is prenylated on the sulfur and then esterificated with methyl on the carboxyl group. The sequence of the K-peptide is H-KISASRKLQLKTC-OH. The peptide had to be reduced at first and then farnesylated, adding the methyl

was the last step to proceed, because the methyl ester could have been frail and its degradation could occur. The peptide was synthesized before in a different laboratory by the same approach, that was used to synthesize the CVLS peptide, but the procedure was done by an automated peptide synthesizer. This machine was not used in the previous case, because the peptide was quite short.

The peptide was at first reduced by DTT and then farnesylated. These reactions were done in the same way as the farnesylation of CVLS. After the confirmation of the structure, the methylation took place.

First the methylation was tried by methyl iodide with pyridine.




But because the compound MS analysis that was done after this reaction did not prove the wanted result, different reaction was chosen.

A simple esterification of the K-peptide by a methanolic solution of hydrochloric acid was performed, but because the peptide was destroyed under these reaction conditions, a more gentle method was used, according to Falick[10]. To an absolutely dry methanol, acetyl chloride was added and the peptide was esterificated with this solution.

## Bovine albumin

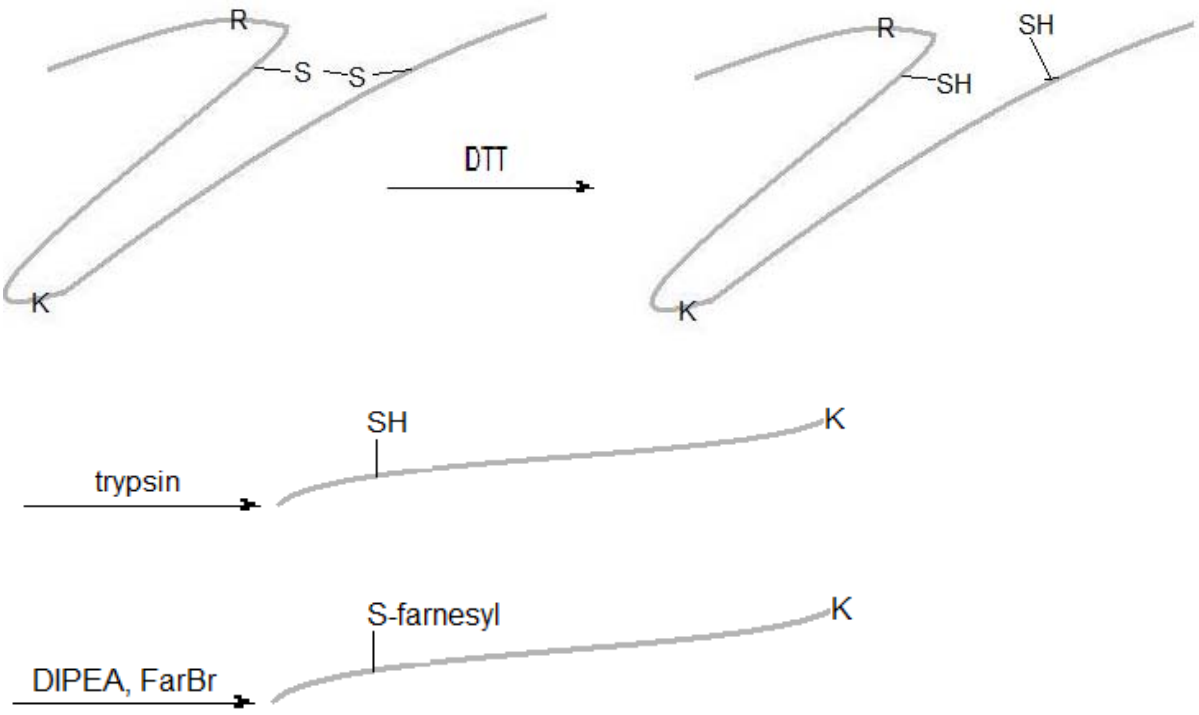
Albumin was chosen as a simple and easily obtainable protein. It was chosen because after the farnesylation it would imitate naturally prenylated proteins, although without the methyl group on the carboxylic end of it and not ending by a cysteine (because trypsin, which was used during the process, does not cleave proteins there). Once the farnesylated peptide could be detected from the spectra, it would not be difficult to change the

parameters in the search programme and add methyl modification as well as farnesyl.

**Bovine albumin farnesylation**

First the albumin was reduced by DTT. Then it was digested by trypsin, which cleaves the proteins on the carboxylic side of arginine and lysine residues. The trypsin had a reductive methylation to minimize its autolysation. Then these peptides were farnesylated by DIPEA and farnesyl bromide again. Not all the cysteines were substituted with the farnesyl moiety; it proved to be useful later, because a separation of farnesylated and non-farnesylated peptides was tried on these.

**Fig. VII: The way to farnesylate proteins**




**Separation of farnesylated and non-farnesylated peptides**

This method should have been developed, in order to see the farnesylated peptides more clearly and with better resolution on the spectra. In a sample made from real cells, there are many different proteins and peptides and only a little amount of them is prenylated, therefore it is important to get rid of the peptides that we are not concerned in and which

could shield the peptides of interest. This method should be as easy and time undemanding as possible. There are some methods, how to separate the prenylated proteins from unprenylated ones and they are usually based on the different lipofility of a normal proteins and proteins with the farnesyl moiety attached. For example separation of prenylated proteins by a surfactant, this has a big disadvantage in the MS analysis afterwards, as the surfactant has to be removed. Other example can be sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which is very time consuming and is also difficult to automate. Therefore we did not want use any special column prior to the HPLC-MS analysis itself and instead tried to separate the peptides by a simple lipo/hydrophilic separation.

The Folch method, which is usually used for separating lipids was used and its modifications. The peptide was diluted in a chloroform/methanol solution and then water was added, which resulted in separation onto to phases, both were analyzed. Another method based on Folch was used - the peptide was diluted in chloroform/isopropanol solution and then separated when ware was added. Isopropanol can be salted out from aqueous solution by adding some inorganic salt; therefore another layer was obtained from the watery phase. Final method, that was used, was separation between ethyl acetate and water.

## MCF- 7 breast cancer cells

The MCF- 7 breast cancer cell line was chosen as a target for whole-cell analysis because they were constantly cultured on the workplace. Also there was a high probability, that they contain some endogenous farnesylated proteins (e.g. H-Ras) in a higher distribution, than in normal cells (as approximately 20% of all cancer is caused by over expressing of H-Ras and similar proteins, containing prenyl). The cells were lysed and the cell membrane and its proteins was the target of the isolation (as the prenylated proteins are anchored there).

The cells were at first lysed by a complex buffer containing sodium chloride to lyse the cells, protease inhibitors cocktail, containing several

protease inhibitors like pepstatin, leupeptin, aprotinin and many more to inhibit the endogenous enzymes that are released from the cells after its disruption. These enzymes would degrade the proteins of interest and would make the further analysis difficult. The buffer also contained deoxycholic acid and lauryl sulfate to solvate the proteins, octyl phenoxylpolyethoxylethanol a nonionic, non-denaturing detergent that breaks the cell membrane. Then after centrifuging and filtering it, the supernatant was denatured by a buffer containing Tris and EDTA to disrupt the hydrogen, hydrophibic and electrostatic bonds. Then the proteins were reduced by TCEP, which is more powerful and also irreversible reductant, compared to DTT. Then the peptides were digested by trypsin and analyzed. The method is a modified procedure after Yokoyama and Zhao[11, 12].

# Experimental part

# Equipment used

HPLC/MS: Thermo Finnigan LTQ – linear ion-trap mass spectrometer coupled with Surveyor

LC pump and autosampler

Balance: Sartorius LP3200D

Analytical balance: Sartorius Genius 215P

Centrifuge: Eppendorf 5415R

Rotary vacuum evaporator: Jouan RC 1010

Nitrogen evaporator: Organomation Association Ltd. N-EVAP112

Lyophilizer: Thermosavant Modulyo D

Desalting pipette tips: Millipore ZipTip

Centrifugal filter device: Millipore Microcon

Chromatographical columns: Phenomenex C18 Gemini 3μ 110 A, 30×2 mm, 3 micron

LC packings A Dionex Company monolithic PS-DVB 200 μm

Zorbax SB300 C8, 3.5 μm, 2.1×50 mm

# Materials

Acetonitrile HPLC grade: Rathburn chemicals Ltd.

Ammonium bicarbonate 99%: Sigma-Aldrich

Ammonium sulfate ultrapure: Merck

O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate: Sigma-Aldrich

Bovine Albumin: Sigma-Aldrich

Cell protein extraction kit: ICAT assay Kit by Applied sciences

Diisopropylethylamine 98%: Sigma-Aldrich

Dimethylformamid 99%: Sigma-Aldrich

Dimethylsulfoxide dried: Rieadel-de Haen

DL-Dithiothreitol: Sigma Aldrich

Ethylacetate 99%: Labscan sciences Ltd.

Ethylenediaminetetraacetate sodium: Merck

trans,trans farnesyl bromide 95%: Sigma-Aldrich

Fmoc-cysteine(StBu):Merck

Fmoc-leucine: Merck

Fmoc-serine (Fmoc-Ser(tBu)-NovaSyn®TGA): Merck

Fmoc-valine: Merck

Formic acid 99%: Riedel-de Haen

Hydrochloric acid 37%: Riedel-de Haen

Chloroform analytical reagent: Labscan Ltd

K-peptide: obtained with kind permission from Professor Alex Azhayev

Mammalian cell lysis kit: Sigma-Aldrich

Methanol HPLC grade: Mallinckrodt Baker B.V.

MCF7 – breast cancer cells: obtained with kind permission from Johanna Kuokkanen

Methyliodide 99%: Fluka

Propan-2-ol: Riedel-de Haen

Pyridine 99%: Riedel-de Haen

Sodium thiosulfate pentahydrate: Merck

Trifluoroacetic acid: Sigma-Aldrich

Triisopropylsilan: Sigma-Aldrich

# CVLS – synthesis and analysis of a simple peptide

## Peptide synthesis

The peptide was prepared by standard solid phase peptide synthesis with Fmoc. The procedure was made manually.

50 mg (40 μmol) of serine resin (Fmoc-Ser(tBu)-NovaSyn®TGA) was given into a tube and washed two times with DMF, then dried by nitrogen. 5 mL of 20% piperidine in DMF was added two times to remove the Fmoc protection group. The resin was then washed three times with DMF.

15 mg (40 μg) of Fmoc-leucine in a tube was preincubated with 0.5 mL of HBTU and 0.5 mL of DIPEA for 5 minutes. The activated amino acid was added into the reaction tube with the resin and left reacting at room temperature for one hour. The liquid was then removed with nitrogen gas and the dipeptide, bonded to the resin was washed three times with DMF. The completeness of the reaction was tried with a Kaiser test, with negative (colorless) result, it was proceeded further. The reaction continued with the removal of the Fmoc protective group from valine and coupling it to the LS-resin reactant in the same manner as before. The final amino acid added was Fmoc-cysteine(StBu). The amount of the material used was 14 mg of Fmoc-valine and 24 mg of Fmoc-cysteine(StBu) (40 μg).

Then 2 mL of the cleavage solution was added to the dry CVLS-resin. The solution was formed of 1.9 mL TFA, 0.06 mL EDT, 0.02 mL triisopropylsilan and 0.02 mL water. The tube with the reactants was incubated for two hours. Afterwards the solution was poured to a tube with cold ether and the peptide crystallized. Ether was separated by centrifugation and water was added to the peptide. The peptide was then lyophilized; the yield was 10 mg of CVLS (60%).

## Identification of CVLS

The identity of the peptide was confirmed by LC-MS. The spectra were showing signs of both CVLS and the oxidized dimer with cystin bonds. Therefore the peptide was reduced: 0.5mL of the peptide solution in

concentration 10 mg/mL was taken to a tube and pH was adjusted from 2 to the pH of 8-9 by ammonia. Then 103 µL of 0.115 M DTT solution (11.86 µmol) was added and kept for 30 minutes at 55°C. A sample was prepared for MS analysis in the concentration 10 µg/mL and the rest of the stock solution was stored in freezer.

**Configuration and method of the analysis:**

Column: Monolithic PS-DVB, 5 cm×200 µm

Flow rate: 150 µL/min reduced to 4 µL/min by a splitter

Gradient and time of elution: 2-60 % acetonitrile in 0.1 % formic acid; 30 minutes

Number of scan events: 1. Full scan from 300 - 1000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

4. Zoom of the most intense ion from the first scan

Capillary voltage: 24 V

The peptide was still dimerised, probably because of the big dilution for the MS sample, where the peptide is not sufficiently under effect of DTT and immediately turns from reduced form into the dimer. Hence the reduction must always take place before other reactions including sulfide groups, if necessary.

**Farnesylation of the CVLS**

1 mg (2.4 µmol) of the peptide, that was reduced before (see above) was evaporated from the solution by nitrogen. To the tube with the dried peptide 135 µL of DMF/DMSO/water (71/25/4) was added and mixed on a tube shaker. Then 15 µL of 0.6 M DIPEA solution (9 µmol) and 15 µL of 0.2 M farnesyl bromide solution (3 µmol) were added. Both of these solutions were prepared by diluting the concentrated DIPEA (98%) or trans,trans- farnesyl bromide (95%) by DMF/DMSO/water. The mixture was shaken up again and

kept at room temperature for 1 hour. The sample for MS analysis was made in concentration 10 μg/mL in 0.2% formic acid. The stock solutions were stored in freezer.

Different concentrations of the reactants were tried in order to raise the yield of the reaction (which was roughly 20%), but none of the reactions had better yields than the one described. Also a temperature of 55°C was tried during the incubation, but no significant change in the yield was accomplished.

**Configuration and method of the analysis:**

Column: C8 column with 3.5 μm particle size and column dimension 50×2.1 mm

Flow rate: 200 μL/min

Gradient and time of elution: 2-76 % acetonitrile in 1 % formic acid; 15 minutes

Number of scan events: 1. Full scan from 600 - 2000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

Capillary voltage: 24 V

## K-peptide – farnesylation, methylation and analysis

The sequence of the K-peptide is H-KISASRKLQLKTC-OH. Several methods of methylation were tried; the two more successful ones will be described below. The peptide was reduced then farnesylated, and then the methylation took place.

500 μg of K-peptide was dissolved in 200 μL of 0.1M ammonium bicarbonate solution, then 60 μL of DTT in concentration 1mg/mL was added and the tube was kept for 45 minutes at 55$^{O}$C. After the reduction, the sample was lyophilized. The dry sample was again diluted in 80 μL of DMF/DMSO (73/27),

10 μL of DIPEA (0.6M) and 10 μL farnesyl bromide solution (0.2M) were added and the mixture was kept at room temperature for 1 hour. The solvents were partly evaporated. To the rest a little amount of water was added and then the peptide was lyophilized.

**$CH_3I$ methylation:** the dried farnesylated peptide was dissolved in 620 μL of dried methanol with 80 μL of pyridine (it was not dissolved perfectly even though it was lyophilized). The reaction took place under argon. 50 μL of methyl iodide was added to the tube and it was stirred in dark for 18 hours. Then the reaction mixture was quenched with 500 μL of 0.1 M sodium thiosulfate, until the yellow color of the reaction mixture disappeared. The samples were prepared directly from the mixture by diluting it with 0.2% formic acid to the concentration 10 μg/mL.

**Esterification:** first an methanolic hydrochloric acid solution was made by adding drop wise 800 μL of acetyl chloride into 5 mL of dry methanol while stirring, though the methanol was dried, the mixture was popping and cracking. Then, to the dried farnesylated K-peptide 60 μL of this methanolic HCl solution was added and left to react for two hours. The sample was also made directly as in the previous method.

**Configuration and method of the analysis:**

Column: C18 column with 3 μm particle size and column dimension 30×2 mm

Flow rate: 200 μL/min

Gradient and time of elution: 8-76 % acetonitrile in 1 % formic acid; 14 minutes

Number of scan events: 1. Full scan from 450 - 2000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

4. Zoom of the most intense ion from the first scan

5. Zoom of the second most intense ion from the first scan

Later, because of difficulties finding the farnesylated and methylated compound, direct MS/MS scan of 847.0 fragments was also incorporated.

Capillary voltage: 24 V

## Bovine albumin – synthetic farnesylation and analysis of a model protein

### Bovine albumin farnesylation

20 mg of bovine albumin (0.3 μmol) was dissolved in 2 mL of 0.1 M ammonium bicarbonate solution. Then 0.2 mL of DTT solution with the concentration 10 mg/mL (13μmol-more than needed to reduce every cysteine in the molecule) was added and incubated for 45 minutes at 55°C. Then 0.55 mL of the solution – the amount according to 5 mg of albumin – was taken into a tube and 0.875ml of trypsin solution (0.2 mg/ml) was added, the tube was kept at 37$^{O}$C for two hours and another portion of the same trypsin solution in the same amount as before was added. The mixture was afterwards incubated for 15 more hours.

0.45 mL of trypsin digested albumin solution (1 mg of the peptides) was then evaporated by nitrogen until only about 30 μL was left in the tube. To this remainder 150 μL of DMF/DMSO (73/27), 20 μL of 0.6 M DIPEA solution and 20 μL of 0.2 M farnesyl bromide solution was added, mixed and left reacting for 1 hour at laboratory temperature.

The sample for MS was made from the final product and from the digested albumin as a comparison. Both were prepared by diluting these with 0.2% formic acid.

The reaction was tried in 8 different ways, changing the amounts of DTT or trypsin, that were used – in almost all of the samples, farnesylated peptides were found. Also the reaction sequence of reduction, farnesylation and tryptic digestion were changed in order to find the right way to proceed with

the reaction, because there is the problem with solvating the peptide, once it is totally dry. Also the farnesylation must be done in a relative lipofilic environment; otherwise two layers would form between water and the farnesyl bromide. Tryptic digestion must then again be done in water. Finally in the sample, prepared by the method that was described previously, suitable farnesylated peptides where found. On these it was possible to explain how do these peptides look like and how to search for them.

**Configuration and method of the analysis:**

Column: Monolithic PS-DVB, 5 cm×200 μm

Flow rate: 200 μL/min reduced to 4 μL/min by a splitter

Gradient and time of elution: 5-63 % acetonitrile in 0.1 % formic acid; 35 minutes

Number of scan events: 1. Full scan from 480 – 2000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

4. Zoom of the most intense ion from the first scan

Capillary voltage: 24 V

## Separation of farnesylated and non-farnesylated peptides

This separation was carried out on farnesylated and tryptic digested bovine albumin. Its preparation was described above. The reaction was done in three different ways in order to obtain more data to compare.

**Folch:** 300 µL of the tryptic digested and farnesylated albumin solution in concentration 1 mg/mL was evaporated on rotary vacuum evaporator. Then 500 µL of chloroform/methanol solution (2/1) was added and agitated in orbital shaker for 20 min. The insoluble particles were removed by centrifugation. To the solution 100 µL of water was added, vortexed and centrifuged for 2 minutes at low speed (2000 rpm), until there were two steady phases. The upper watery phase was removed and diluted into a sample and the lower chloroform layer was evaporated and then diluted into a watery sample with 0.2% formic acid.

**Chloroform/isopropanol/water (changed Folch):** 300 µL of farnesylated peptides solution was treated in the same way as in the first reaction that means evaporated and then again dissolved. The dry peptides were dissolved in 500 µL of chloroform/propan-2-ol (2/1). Then 100 µL of water was added, vortexed and centrifuged. Two phases were formed, one was chloroform with the majority of the isopropanol and the other was the watery phase. The two phases were separated and both divided to two other, one of which was kept for analysis and the other one to continue the experiment. The theory was to get another phase by salting out the isopropanol from the solution by ammonium sulfate. The water phase did not do anything after adding the concentrated solution of ammonium sulfate(as expected, because majority of the isopropanol probably stayed in the chloroform, but the chloroformic phase developed two layers after centrifuging for some time at 4$^{O}$C. The interface was quickly disappearing, while the mixture was becoming warmer. The isopropanol/water layer was taken and desalted with MILLIPORE ZipTip desalter – pipette tips for sample preparation. Three samples were made for MS analysis – two from the first separation (chloroformic and aqueous phase) and one from the desalted isopropanol.

**Ethyl acetate/water:** 300 µL of the peptide solution was evaporated the same way as in the Folch method, then 200 µL of water was added and the tube was agitated for some time, then the mixture was centrifuged to remove the insoluble particles. To the clear water solution, 200 µL of ethyl acetate was

added and shaken. The lower watery phase was diluted on a sample, while the upper ethyl acetate phase was evaporated and then diluted into a sample with 0.2% formic acid.

**Configuration and method of the analysis:**

Column: Monolithic PS-DVB, 5 cm×200 μm

Flow rate: 180 μL/min reduced to 4 μL/min by a splitter

Gradient and time of elution: 5 – 54 % acetonitrile in 0.1 % formic acid; 35 minutes

Number of scan events: 1. Full scan from 400 – 2000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

4. Scan events of 2. and 3. was repeated for the top three peaks

Capillary voltage: 43 V

## MCF- 7 breast cancer cells

## Isolation and analysis of cell membrane proteins

The cells were taken out of the growing medium and washed with PBS. Then lyophilized and stored at -80$^{O}$C (about 4*10$^{6}$ of cells was used). The cells were then resuspended in 1 mL of lysis buffer (Tris-EDTA, NaCl, SDS, DOC, Igepal CA 630 and protease inhibitors cocktail), vortexed for several minutes and then centrifuged for 10 minutes at 12000 g. The cell debris was pelleted and the supernatant (containing the solvated proteins) was removed to a chilled tube and given on ice. 200 μL was then given into Microcon centrifugal filter device with a 30000 Da mesh and centrifuged at 13200 rpm for two hours.

The filtrate was denatured and tryptic digested, ICAT Kit from Applied Sciences was used. 20 μL (roughly 20 μg of proteins) was diluted with the denaturing buffer (50 mM Tris, 0.1% SDS) to 100 μL, then 2 μL of the reducing agent (50 mM TCEP) was added. The sample was boiled for ten minutes and allowed to cool to the room temperature. 200 μL of trypsin solution (25 μg) was added and the sample was incubated for 18 hours. The MS sample was made afterward by diluting in 0.1% HCOOH.

**Configuration and method of the analysis:**

Column: Monolithic PS-DVB, 5 cm×200 μm

Flow rate: 180 μL/min reduced to 4 μL/min by a splitter

Gradient and time of elution: 5 - 54 % acetonitrile in 0.1 % formic acid; 35 minutes

Number of scan events: 1. Full scan from 400 - 2000 m/z

2. MS/MS of the most intense ion from the first scan

3. MS/MS of the second most intense ion from the first scan

4. Scan events of 2. and 3. was repeated for the top three peaks

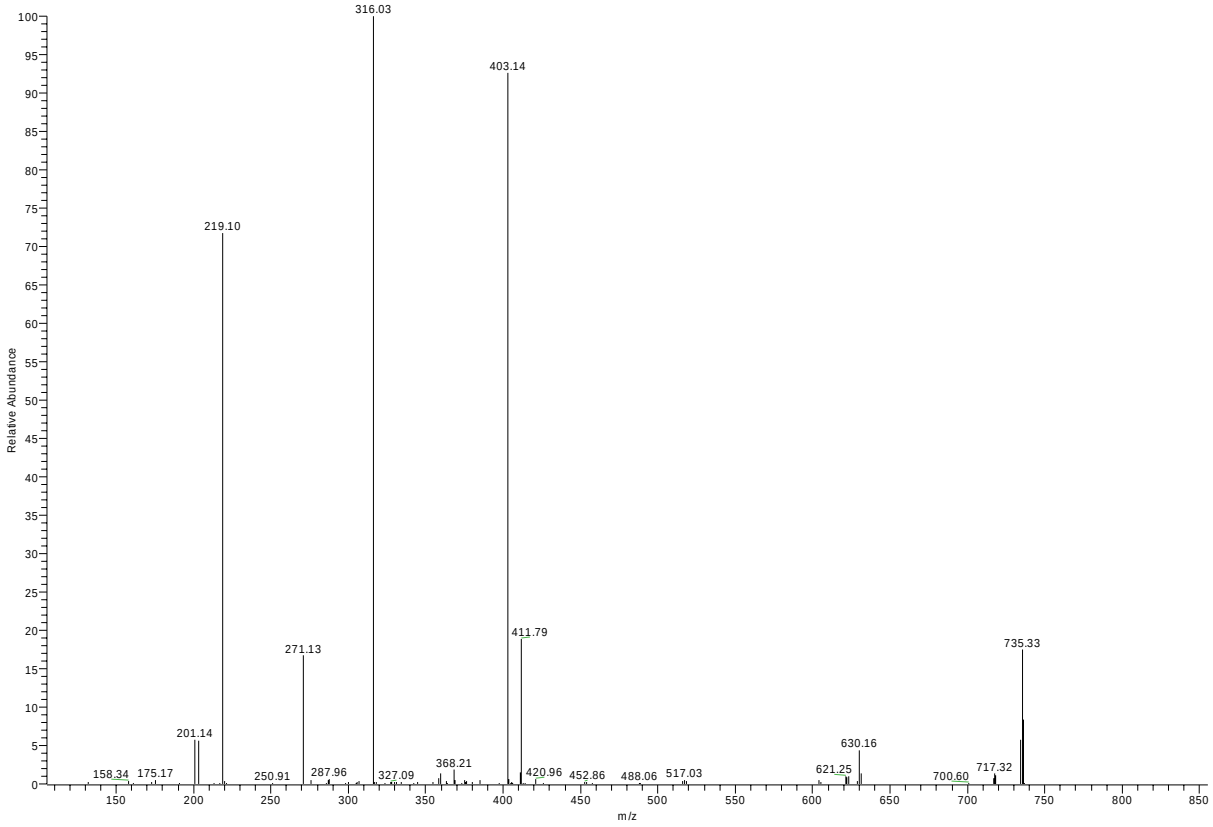
Capillary voltage: 43 V

# Results and discussion

## CVLS spectra interpretation

In the MS spectra of native CVLS, two major peaks were observed - 421.2 indicates CVLS and 839.3 for oxidized CVLS dimer. A MS/MS data gives us a clear information about the primary structure with 201 peak for a y-ion (C-terminus of the cleaved peptide bond) of LS with a neutral loss of a molecule of water, 203 as a b-ion (N-terminus of the cleaved peptide bond) CV, 219 as a y-ion LS, 316 as b-ion CVL and 403 as a mass of the full molecule with the loss of water (see Fig. 1).

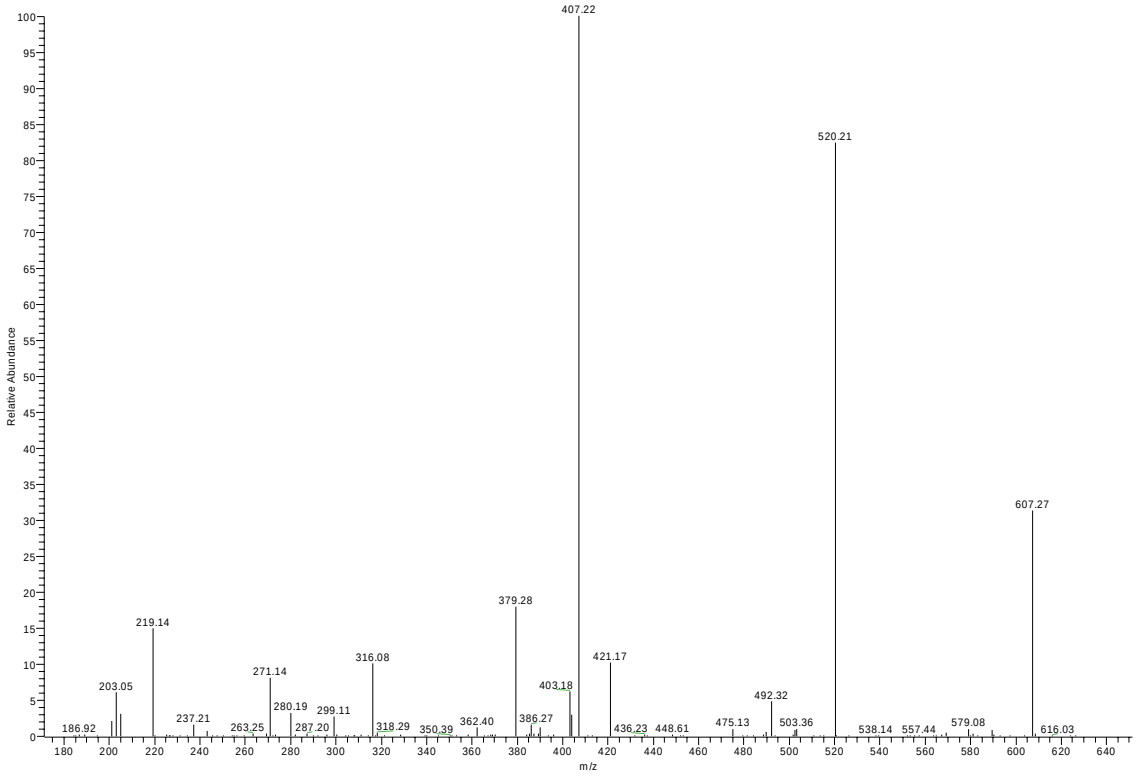
**Fig. 1: MS/MS spectrum of CVLS (parent ion 421)**




The major peak of farnesylated CVLS was 626.4 as for mono molecular mass of the molecule. By the MS/MS spectra the successful farnesylation was confirmed. The peaks can be analyzed as follows: 203 CV fragment, the same as on the spectra of native CVLS, but in this case it is in fact an internal fragment with the loss of the farnesyl moiety; 219 an Y-ion of LS; 316 CVL internal fragment; 379 an a-ion Far-CV (a-ion is an N-terminal ion with the loss

of complete functional group – peptide bond); 403 for CVLS with the loss of molecule of water; 407 y-ion VLS; 421 CVLS without farnesyl; 492 a-ion Far-CVL; 520 b-ion Far-CVL; 607 basic structure with the loss of water again (see Fig.2).

**Fig. 2: MS/MS spectrum of Far-CVLS (parent ion 626)**

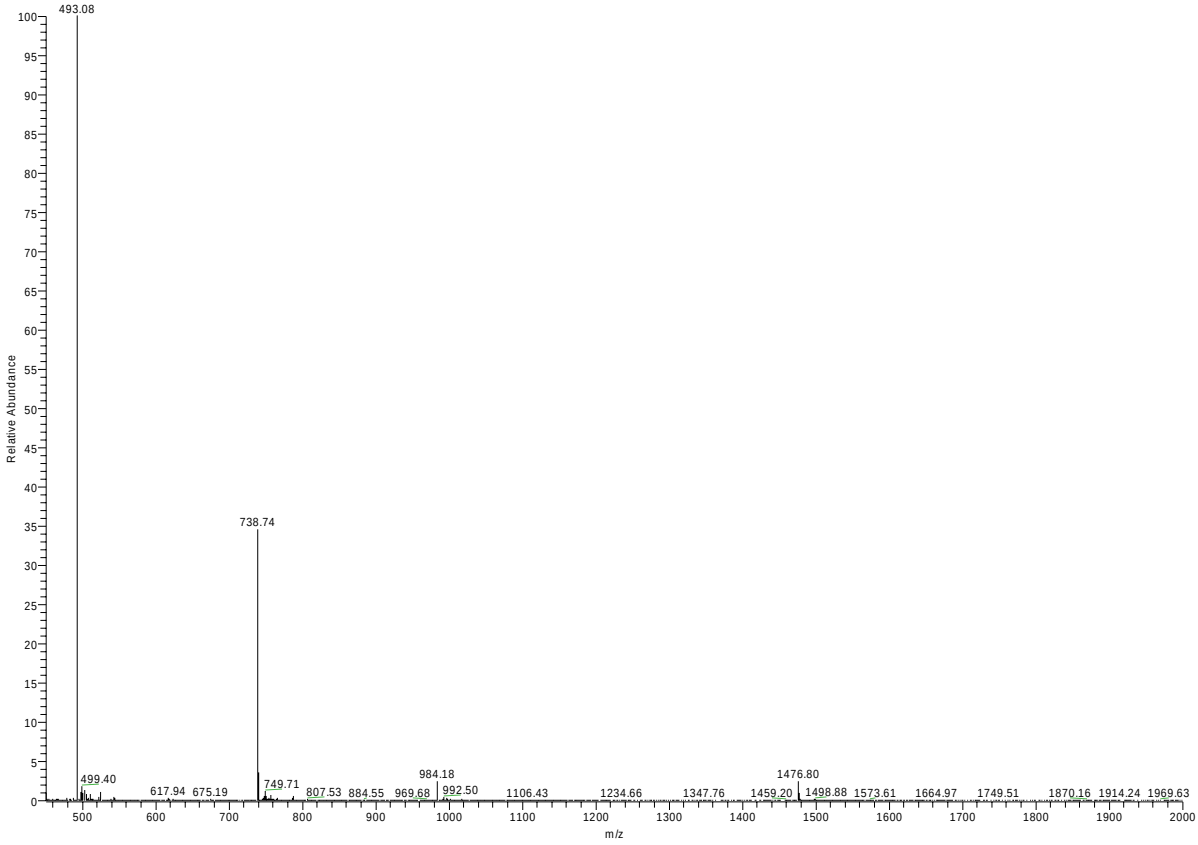



The structure of the farnesylated CVLS peptide was confirmed, but the exact state of the farnesyl moiety, when leaving the molecule and its cleavage could not be pointed out from this data.

## K-peptide spectra interpretation

The K- peptide was identified as KISASRKLQLKTC, because it is a long peptide, it had more, than one charge on its chain. This can be clearly seen on the full MS spectra in Fig.3. Peak 1476 stands for the peptide with one charge, whereas 738 is double-charged and 493 triple-charged molecule.

**Fig. 3: Full MS spectrum of K-peptide**




Complete primary sequence of the peptide was confirmed by MS/MS spectrum of the second most intense ion, which was in this case 738. We can clearly see several peaks: 351 for y-ion KTC; 464 y-ion LKTC; 507 b-ion KISASRKLQ, which is double-charged – this occurs because the parent ion also had more than one charge, same fragment with only one charge is seen also under 1012; 564 double-charged b-ion KISASRKLQL, single-charged under 1126; 618 double-charged y-ion SASRKLQLKTC, single-charged under 1235; 627 double-charged b-ion KISASRKLQLK; 660 internal fragment KLQLKT with a loss of water and two molecules of ammonia; 678 b-ion KISASRKLQLKT; 771b-ion KISASRK; 884 b-ion KISASRKL (see Fig. 4).

**Fig. 4: MS/MS spectrum of K-peptide (parent ion 738)**

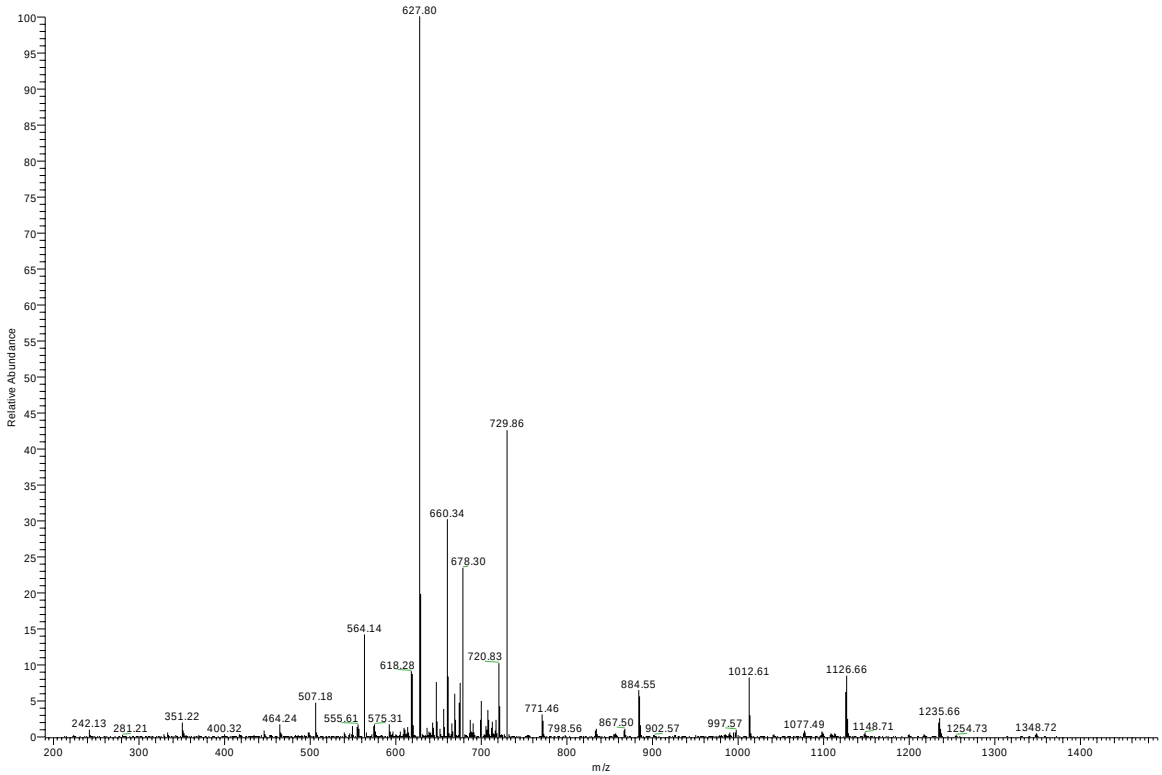
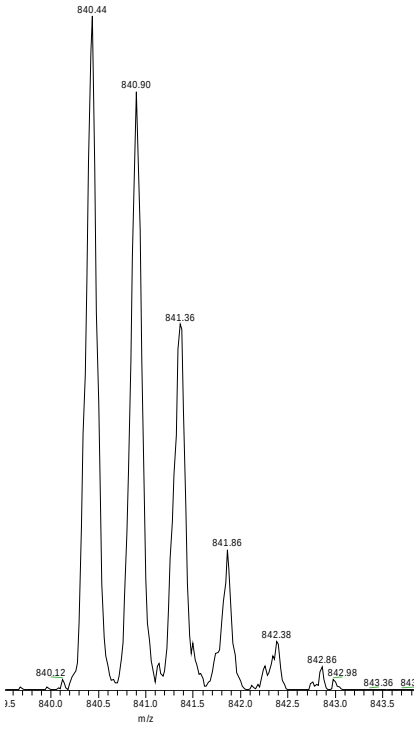



**Fig. 5**

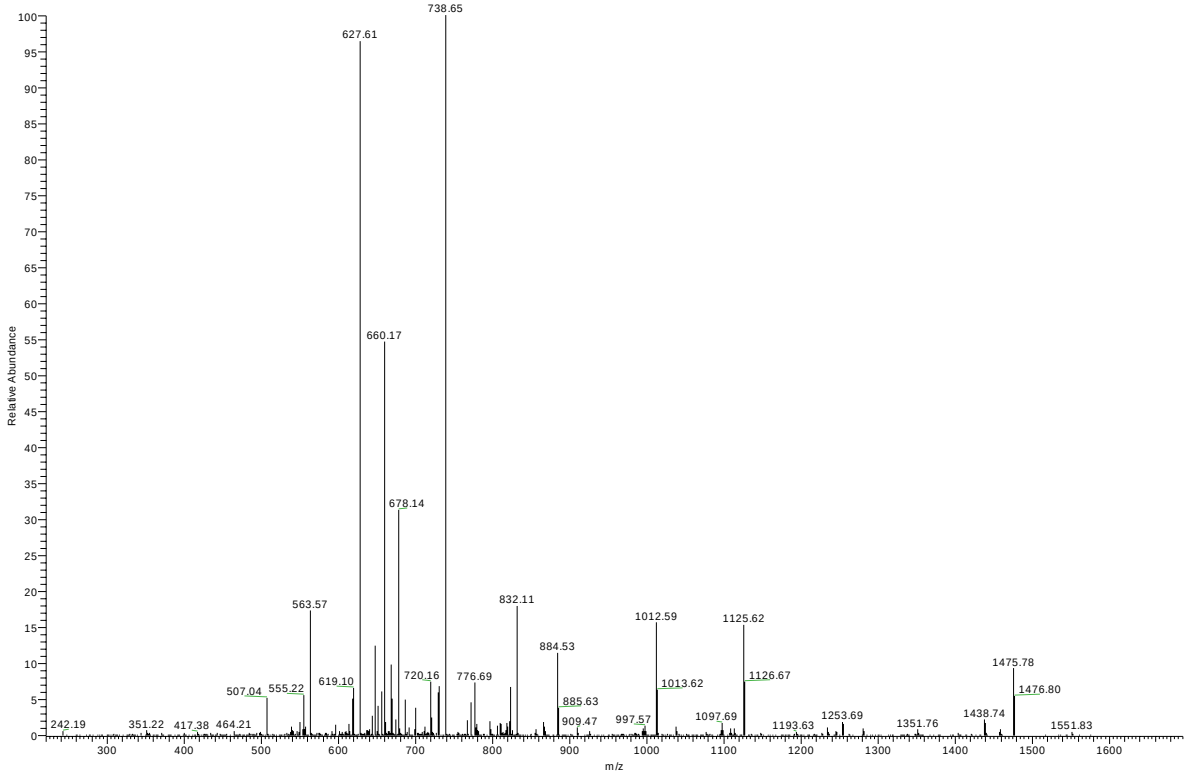



On Fig. 5 , there is a zoom MS spectrum of double charged parent ion of farnesylated K-peptide, it is an example how to predict the number of charges on the fragment – the peaks have a m/z distance of one half, which means that the fragment has two charges. Similarly m/z difference of one means only one charge and one third three charges.

In the MS/MS spectrum of farnesylated K-peptide from the 840 parent ion, there are the same peaks as in the previous spectrum of bare K-peptide, but also some peaks of y-ions, which are containing the attached farnesyl: 1193 – RKLQLKTFar-C; 1351 – ASRKLQLKTFar-C; 1438 – SASRKLQLKTFar-C; 1552 –

ISASRKLQLKTFar-C. However, we cannot see the cleaved farnesyl moiety (as a peak 205) or figure out the cleavage mechanism (see Fig. 6).

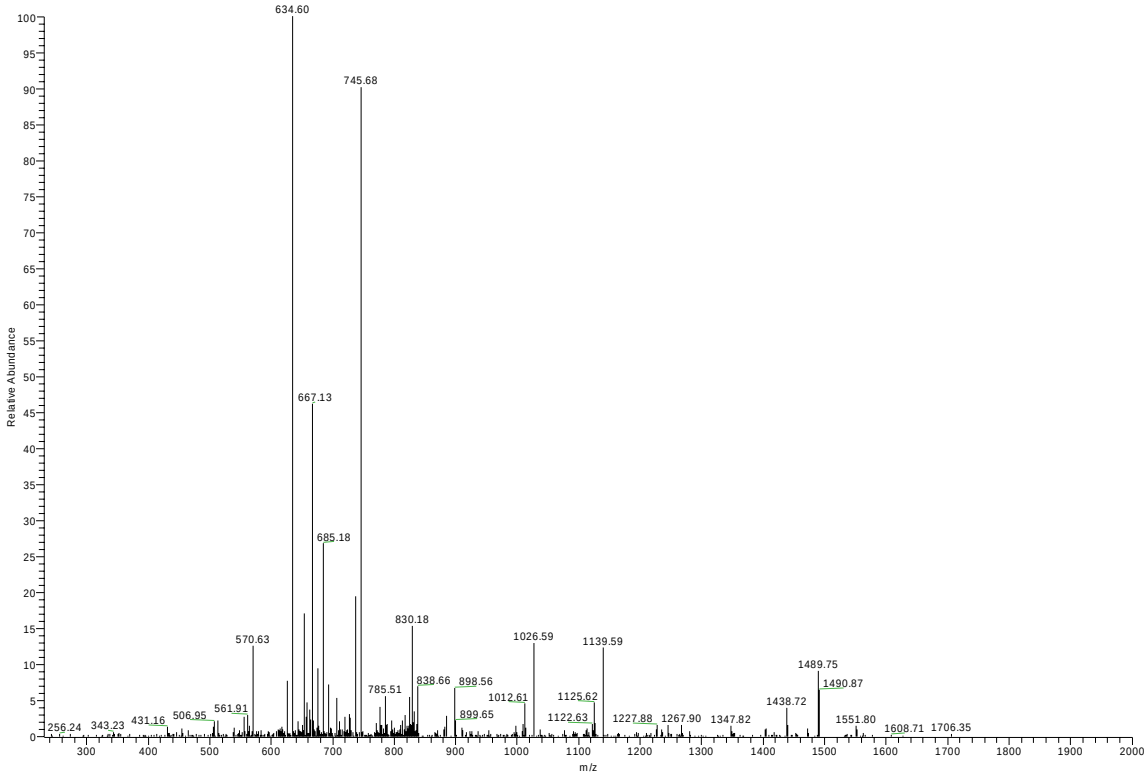
**Fig. 6: MS/MS spectrum of farnesylated K-peptide (parent ion 840)**




The spectra after the $CH_3I$ methylation were showing signs of methyls on the molecule of the K-peptide, but sadly no clear sign of methyl ester on the terminal cysteine. Instead the methylation was probably somewhere on serine or threonine.

In Fig. 7 we can see again similar peaks as on the previous two MS/MS spectra. The most important from the different ions is 745 as for double-charged methylated K-peptide (1489 is the same, but single-charged), which underwent the $MS^3$ breakdown afterwards. There are several y-ions, but all of them are without the methylation. B-ions are 634 a double-charged KISASRKLQLK with a methyl; 685 double-charged KISASRKLQLKT with a methyl; 785 KISASRK with methyl; 898 KISASRKL with methyl; 1026 KISASRKLQ with a methyl, there is also a peak 1012 for the same fragment without the methyl; 1139 KISASRKLQL with methyl and 1125 without it. From these and results we can deduce, that the K-peptide was not esterificated on cysteine, as was intended.

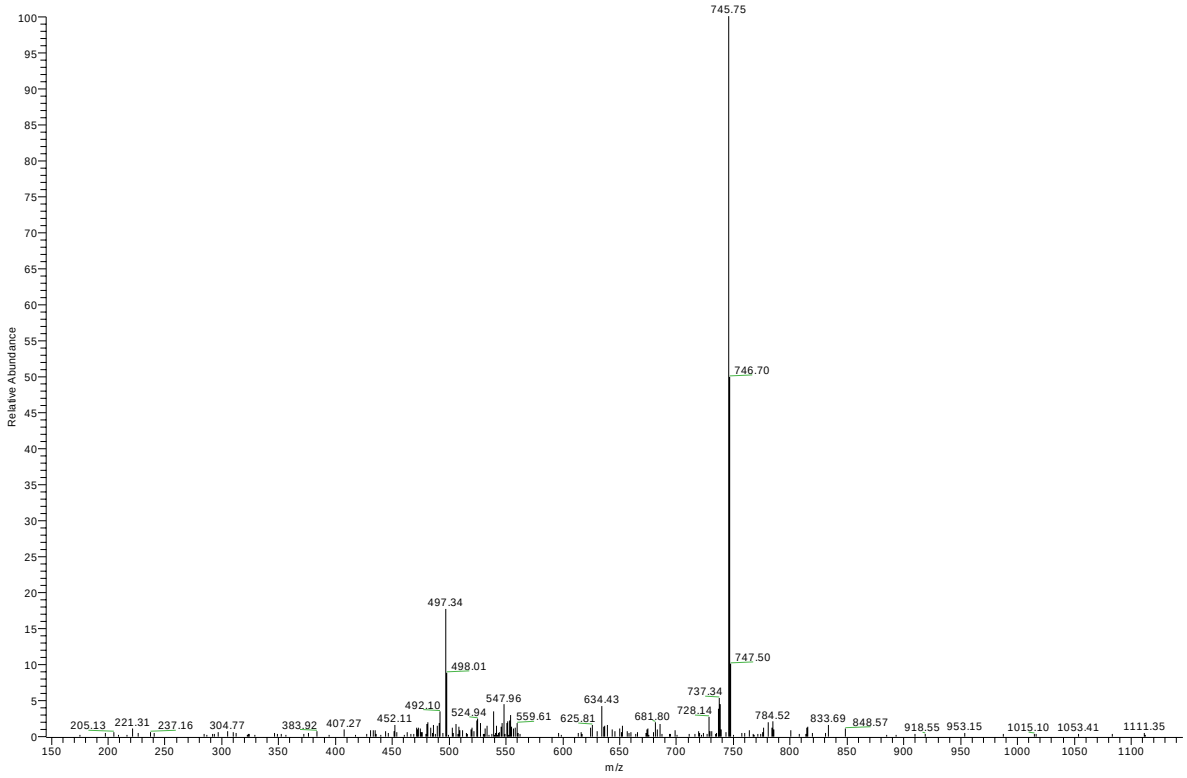
**Fig. 7: MS/MS spectrum of K-peptide after $CH_3I$ methylation (parent ion 847)**




In the spectra of the K-peptide that was esterificated, two peaks of the product were found – triple charged 566 and double charged 847.

In the MS/MS spectrum of 566 ion there are two major peaks 497 and 745, which both result from cleavage of the farnesyl moiety from the methylated K-peptide, one is triple-charged and the other double-charged. There is also a slight 205 peak, which can be interpreted as farnesyl (see Fig 8). A $MS^3$ analysis was made from the 745 peak, but definite conclusion, whether the peptide was methylated on cysteine or somewhere else in the molecule, could not have been carried out. Although uncertain about the result and proper structure of the peptide, I have figured out how to search for peptides like this and at least found out, that the farnesyl moiety is cleaved each time at first induced collision, while the methyl moiety stays attached to the ions.

**Fig. 8: MS/MS spectrum of K-peptide after esterification (parent ion 566)**

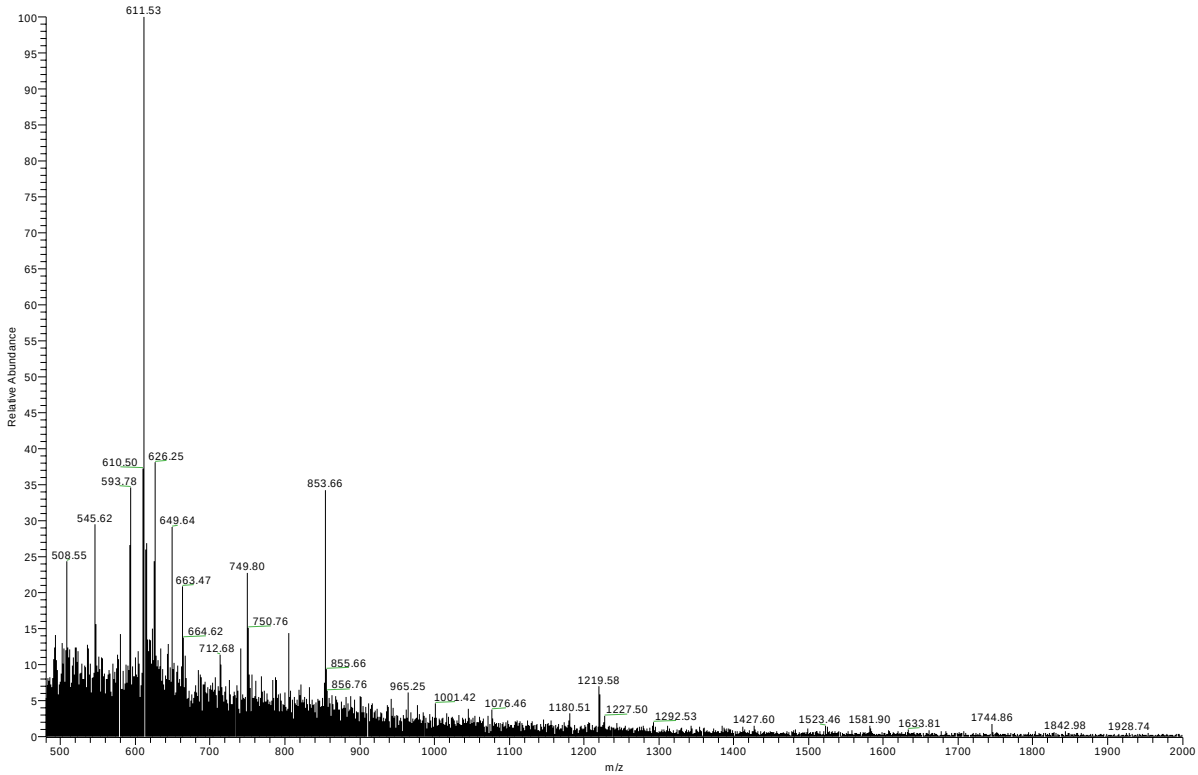



## Bovine albumin spectra interpretation

Bovine serum albumin is a common protein with molecular weight 69.3 kDa and consisting of 607 amino acids of which 34 are cysteines – the possible targets for farnesylation. The data from the MS-measuring were analyzed by Bioworks Browser and 8 peptide fragments with farnesylated cysteine were found by the programme along with numerous other peptides, characterising bovine albumin. All these fragments are products of the digestion with trypsin, therefore their sequence always end with arginine or lysine.For the identification and cleavage interpretation, peptide SHFar-CIAEVEK was chosen, because of its suitable length.

The peak of interest is in this case 611.6(610.3 monoisotopic, see Fig. 9), which represents double-charged peptide SHFar-CIAEVEK that has monoisotopic molecular weight 1219.

**Fig. 9: Full MS spectrum of farnesylated bovine albumin**




The MS/MS spectrum of the peak 611 confirms its identity and among other more complicated ions, that were formed by losses of molecules of water and ammonia or internal fragmentation, it shows these interesting peaks: 225 b-ion SH; 276 y-ion EK; 375 y-ion VEK; 576 y-ion AEVEK; 646 b-ion SHFar-CI; 688 y-ion IAEVEK; 792 y-ion CIAEVEK; 846 b-ion SHFar-CIAE; 945 b-ion SHFar-CIAEV; 996 y-ion Far-CIAEVEK; 1074 b-ion SHfar-CIAEVE. The most important in the spectrum are peaks 508 and 1016, both stand for the peptide itself – SHCIAEVEK- without the farnesyl moiety; double- and single-charged respectively (see Fig. 10). From these two, it was possible to figure out the cleavage mechanism and the form of farnesyl, when leaving the peptide during the reaction. Important thing to see, when doing so was to view the zoom spectra of both peaks and also the peak 611 see Fig. 11).

**Fig. 10: MS/MS spectrum of farnesylated bovine albumin (parent ion 611)**

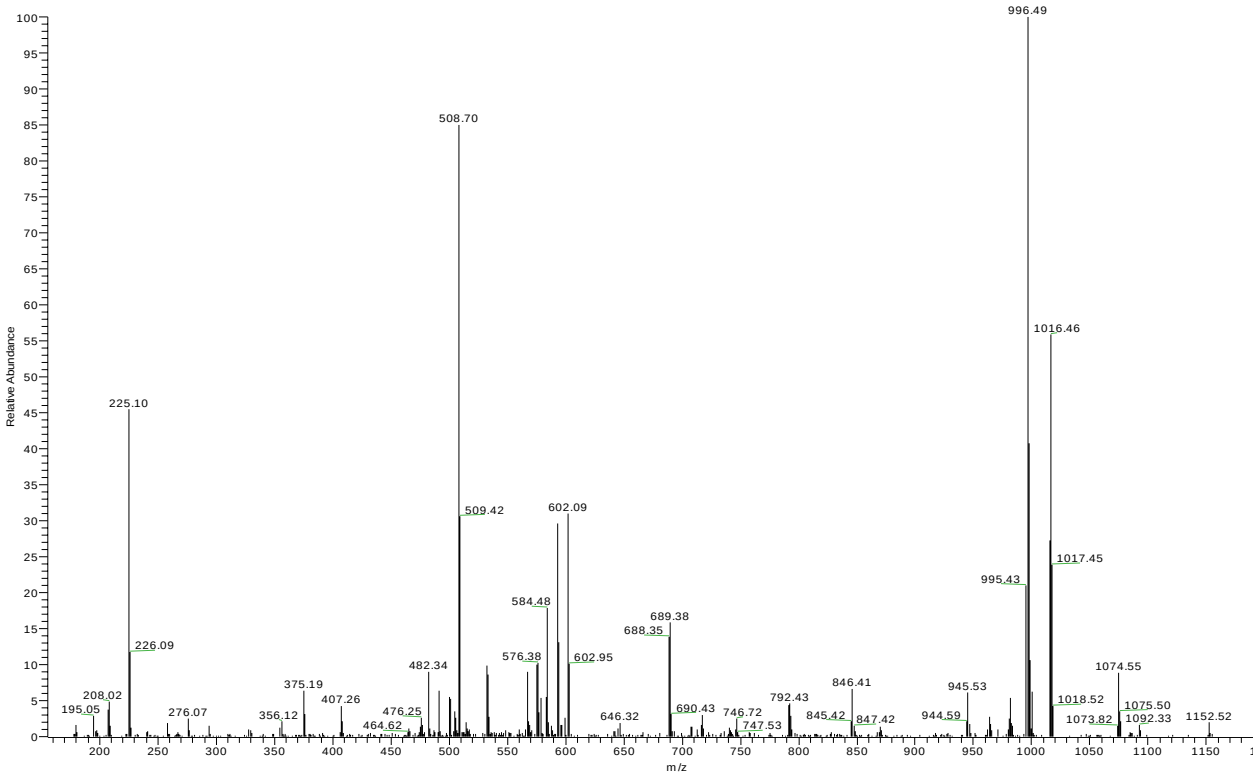
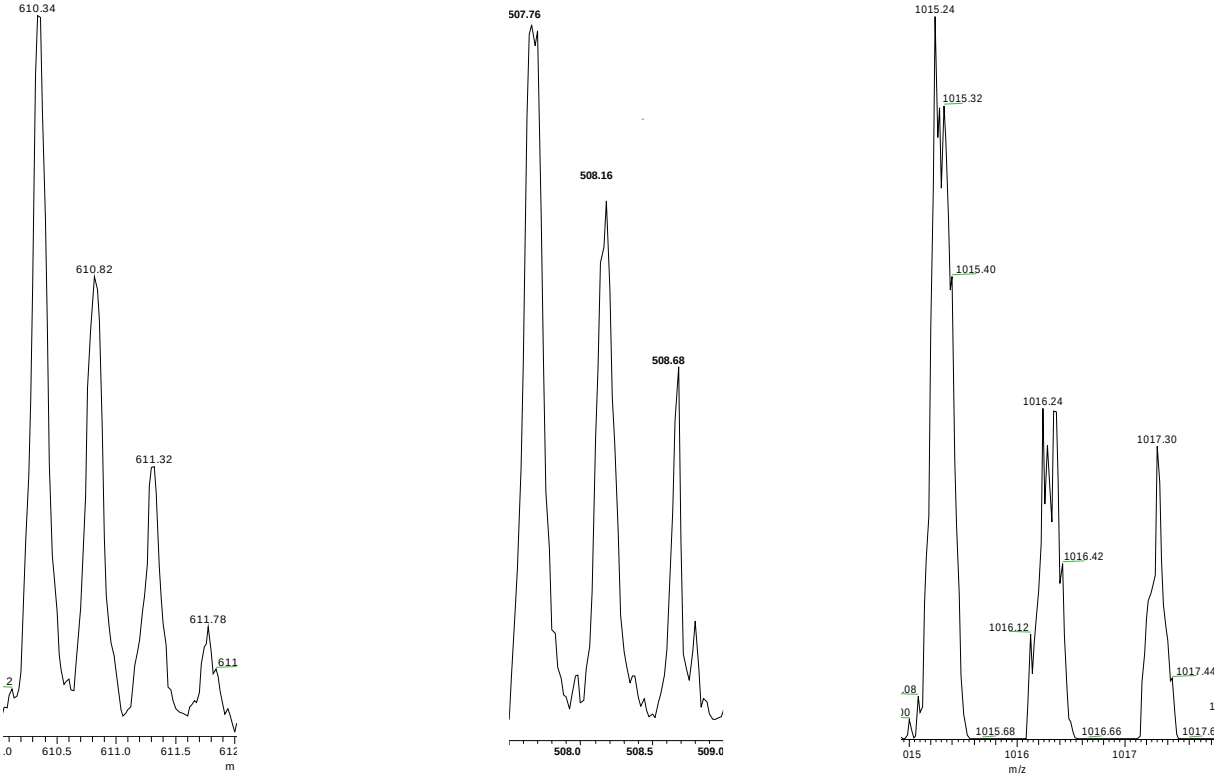


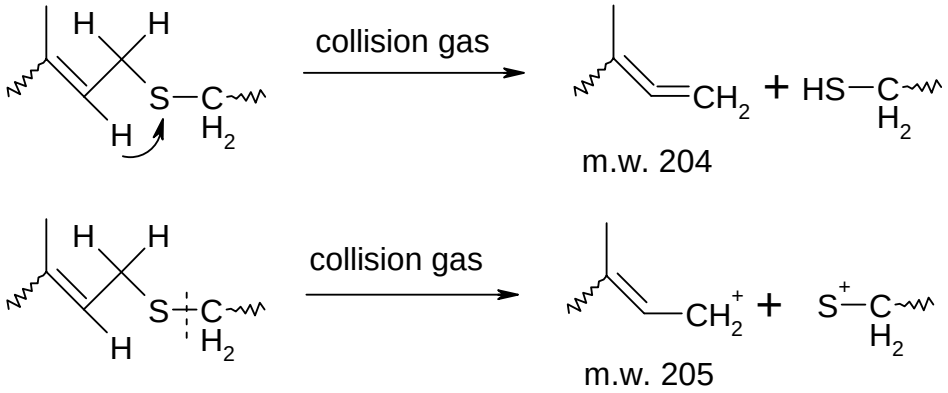

**Fig. 11: Zoom MS spectra of SHCIAEVEK peptide fragment**




610 as the base peak means a total physical mass of 1219 (2×610-1 proton), because it is double charged. The 507 peak means in fact, that it has

the mass one unit lower than the 1015 single-charged peptide. This can only be explained by different ion production. The double charged 507(508) peak is formed by a homo-cleavage between the farnesyl and cysteine, while both moieties remain charged and farnesyl has a mass of 205. The peptide residue has one new charge on sulfur now. The 1015 peak is formed by a hetero-cleavage with neutral loss, the farnesyl moiety looses one hydrogen (probably from the second carbon) to the peptide, which forms the –SH group on cysteine. Farnesyl has now mass of 204, a new double bond and possibly rearranges its molecule.

**Fig. 12: Proposed cleavage mechanism during the analysis**




## Separation of farnesylated and non-farnesylated peptides

**Folch:** An interesting peptide has been found in both samples – a relatively hydrophobic peptide LGEYGFQNALIVR (double-charged peak 740).The main reason why it was in both samples was that in the chloroformic sample, the peptide was farnesylated (double-charged peak 842.5). The peptide does not contain cysteine and therefore is not so interesting for us, but it is a proof that this sort of separation may work. It also shows us that the farnesylation by the used chemicals is not specific on cysteine, but that was also not requested. There was also a number of other peptides in the chloroformic phase, but none was of any interest.

In the water phase much more peptides were found, however not the SHFar-CIAEVEK, which was studied before. In spite of a thorough search only two peptides with a cysteine farnesylation were found – YIFar-CDNQDTISSK. It

has a double-charged parent ion of 796 (see Fig. 13). The other one found was RPFar-CFSALTPDETYVPK with a triple-charged peak 677. Because of its shorter length only YIFar-CDNQDTISSK has been studied closer, after the MS/MS analysis it produces specific peaks: 277 b-ion YI; 321 y-ion SSK; 434 y-ion ISSK; 535 y-ion TISSK; 650 y-ion DTISSK; 778 y-ion QDTISSK; 892 y-ion NQDTISSK; 941b-ion YIFar-CDNQ; 1007 y-ion DNQDTISSK; 1110 y-ion CDNQDTISSK; 1157 b-ion YIFar-CDNQDT; 1270 b-ion YIFar-CDNQDTI; 1314 y-ion Far-CDNQDTISSK; 1444 b-ion b-ion YIFar-CDNQDTISS (see Fig 14). There are also two typical peaks for farnesylated peptides 693 and 1386, which stand for double-charged and single-charged defarnesylated peptides, created by the mechanism that was described earlier.

**Fig. 13: Full MS spectrum of the water phase of farnesylated albumin after Folch**

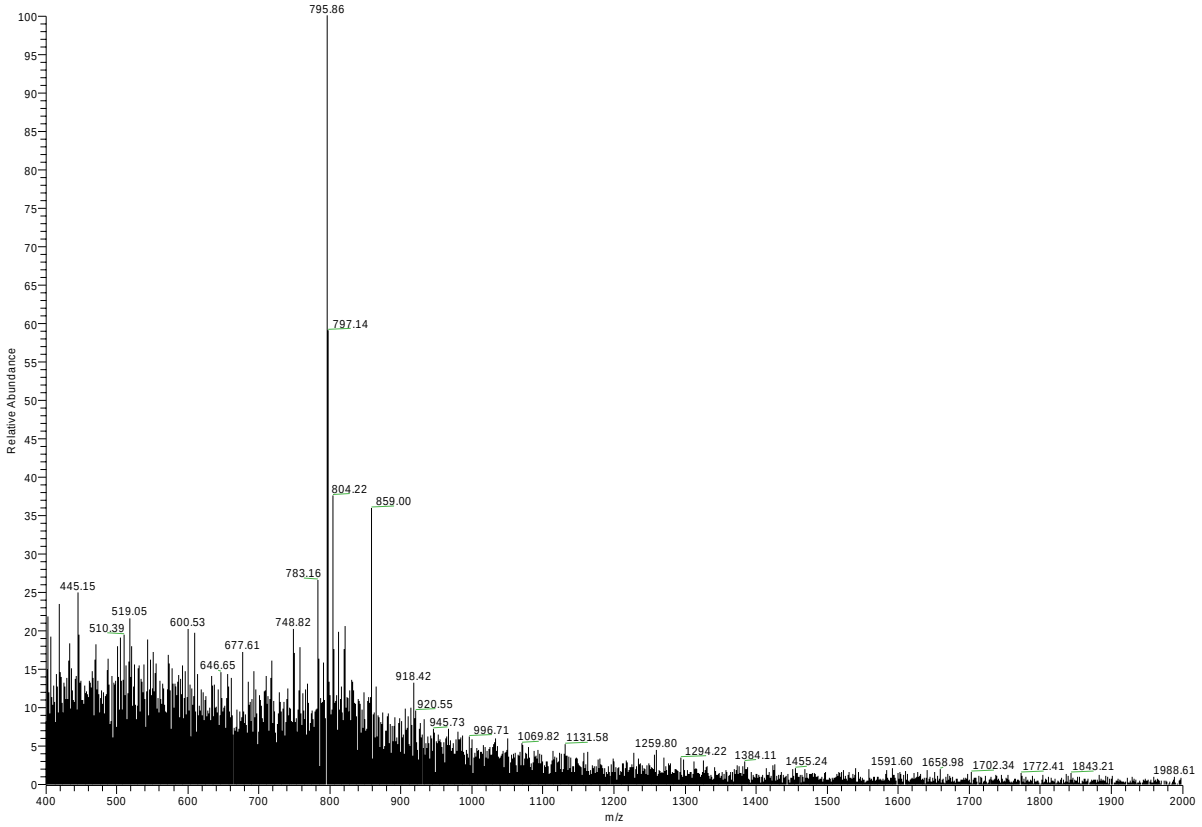
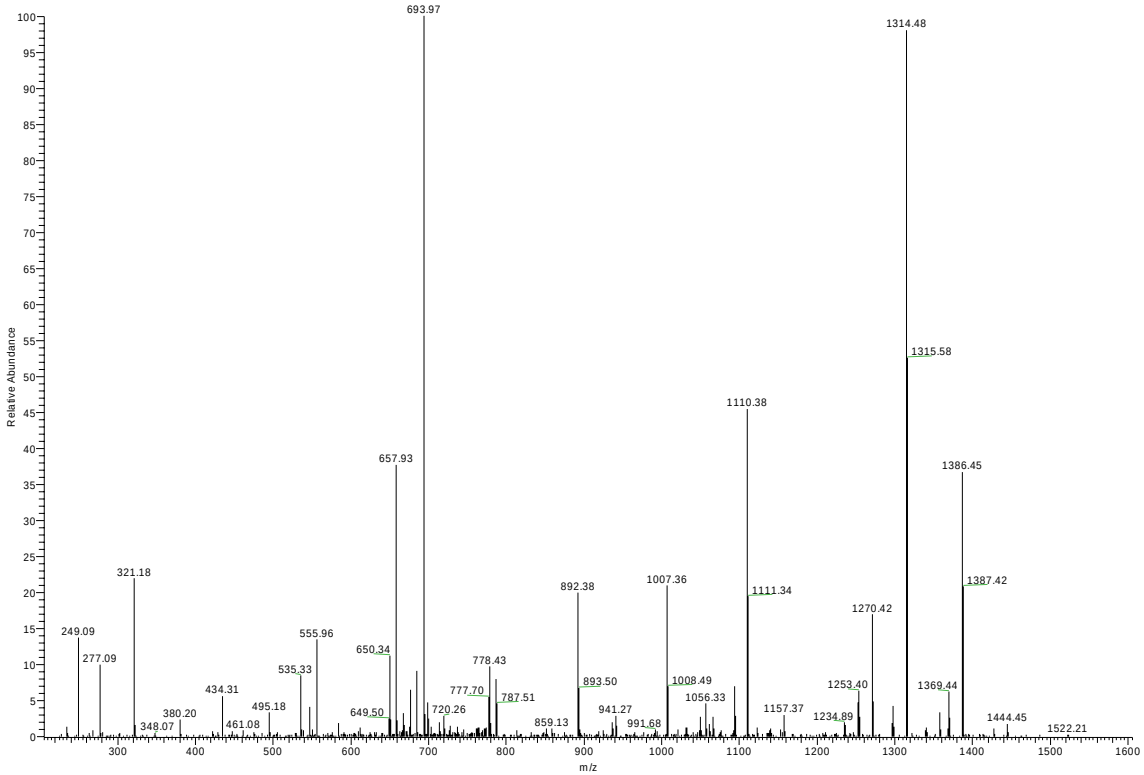


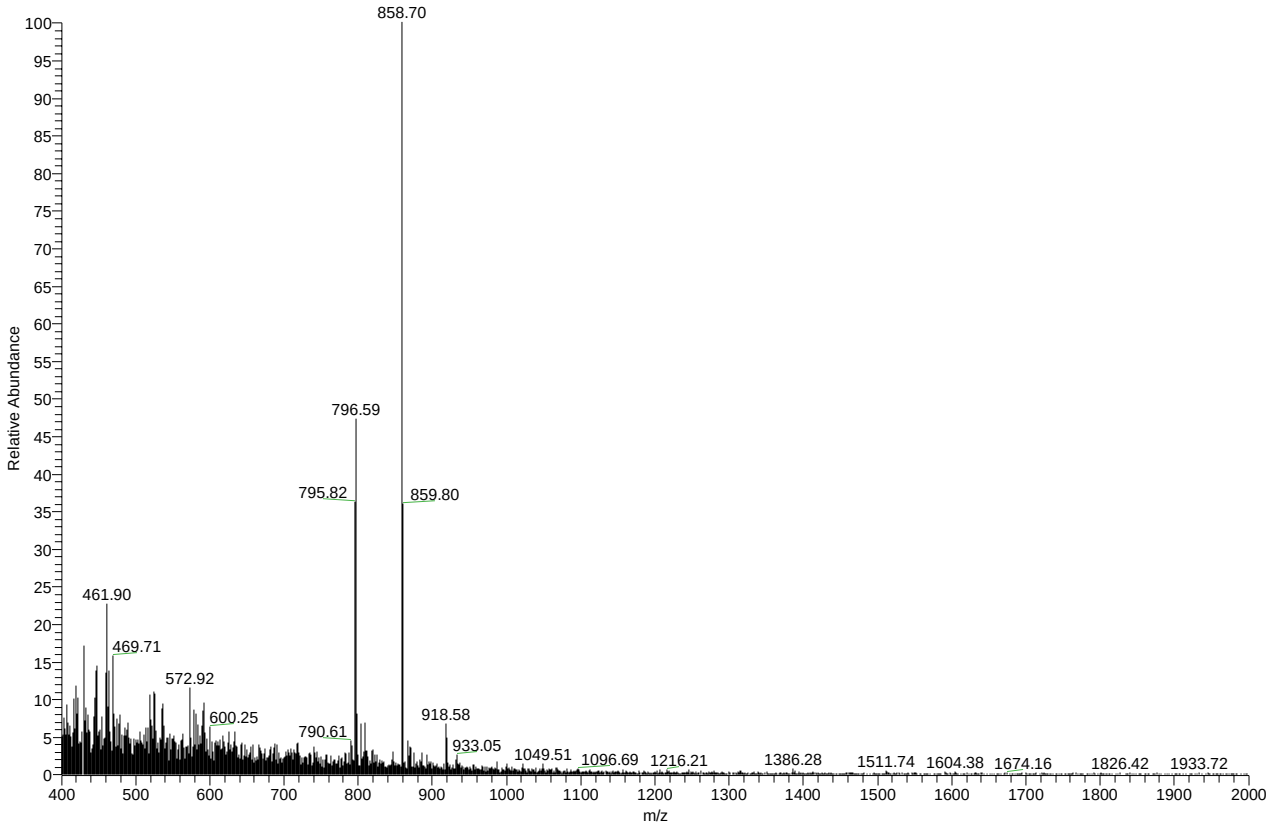
**Fig. 14: MS/MS spectrum of YIFar-CDNQDTISSK peptide (parent ion 796)**




**Chloroform/isopropanol/water:** In the chloroformic layer was again found the peptide LGEYGFQNALIVR as well as in the water layer, but this time the peptide was not farnesylated in the chloroformic layer. Otherwise there were no interesting peptides found in this phase. In the water phase which resulted from the isopropanol salting-out from chloroform, there were only some unknown impurities and residues, no peptide at all could have been identified.

The water phase from this separation was very similar to the water phase from Folch. Only two farnesylated peptides were found among other non-farnesylated ones, again it was YIFar-CDNQDTISSK and RPFar-CFSALTPDETYVPK. Major difference between Folch and this separation was the intensity of the parent ions e.g. in the chloroform/isopropanol/water separation the 796 peak of the peptide was only the second most intense ion at its retention time (see Fig. 15), whereas during Folch it was the most intense ion.

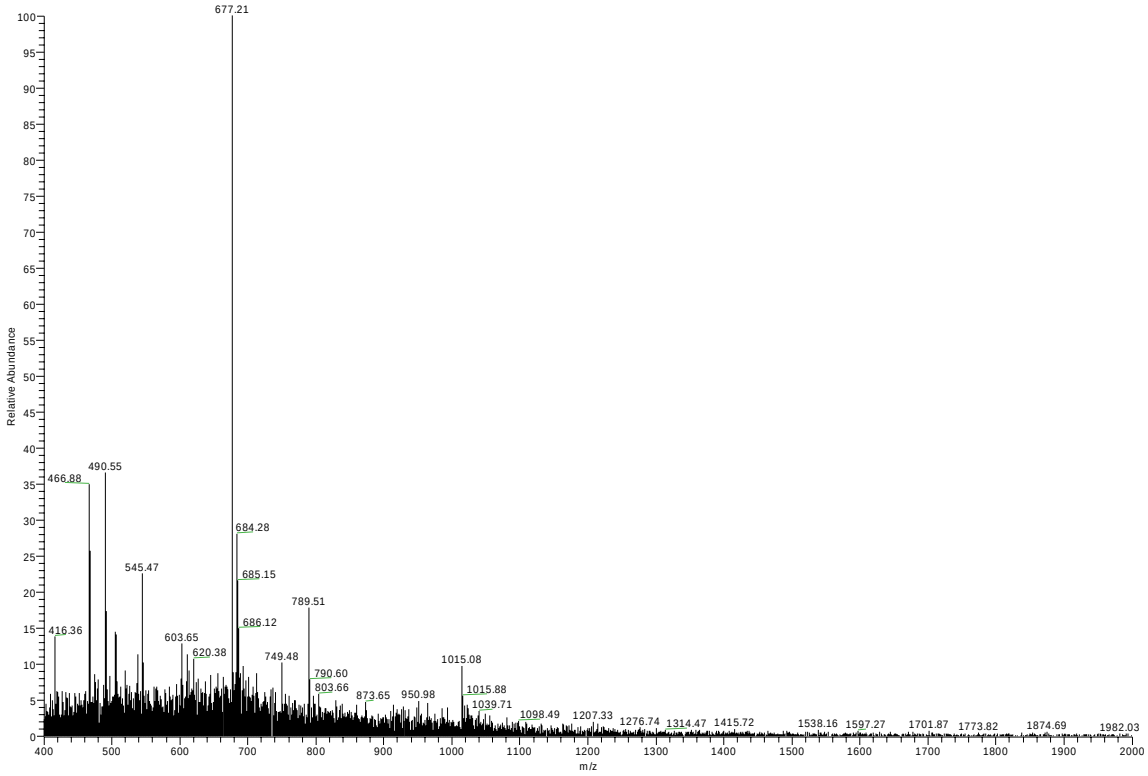
**Fig. 15: Full MS spectrum of the water phase of farnesylated albumin after chloroform/isopropanol/water separation**




**Ethyl acetate/water:** The spectra of ethyl acetate phase were not showing any signs of peptides at all only impurities again. This is quite legitimate, because even the most hydrophobic peptide would probably not dissolve in ethyl acetate.

The water phase should therefore contain all the peptides, but from the farnesylated YIFar-CDNQDTISSK was not found and only RPFar-CFSALTPDETYVPK as triple-charged 677 peak was seen (see Fig. 16). Also the peptide LGEYGFQNALIVR both farnesylated and non-farnesylated and several others were observed.

**Fig. 16: Full MS spectrum of the water phase of farnesylated albumin after ethyl acetate/water separation**




The chromatograms of the measurings are offering another set of information about the distribution of the peptides and their retention times. On Fig. 17 there is a chromatogram of farnesylated peptides from bovine albumin before any separation took place. We can see that the peptides (their retention times are almost the same as in Fig. 18) are distributed in maximum of 4 % relative abundance, because there is a lot of lipophilic impurities washed out in the end of the programme. Fig. 18 represents the chromatogram of a water phase (in which most of the peptides are) sample after the separation (all three cases of separation have similar chromatogram). It is evident, that relative abundance of peptides is approximately five times higher than in the previous figure, because the lipophilic residues were separate into chloroform (ethyl acetate) in quantity. We can also see, that the peptides rarely have clear peaks (only 740), therefore it is necessary to search for them with a bigger effort.

**Fig. 17: Chromatogram of a bovine albumin sample without any separation**

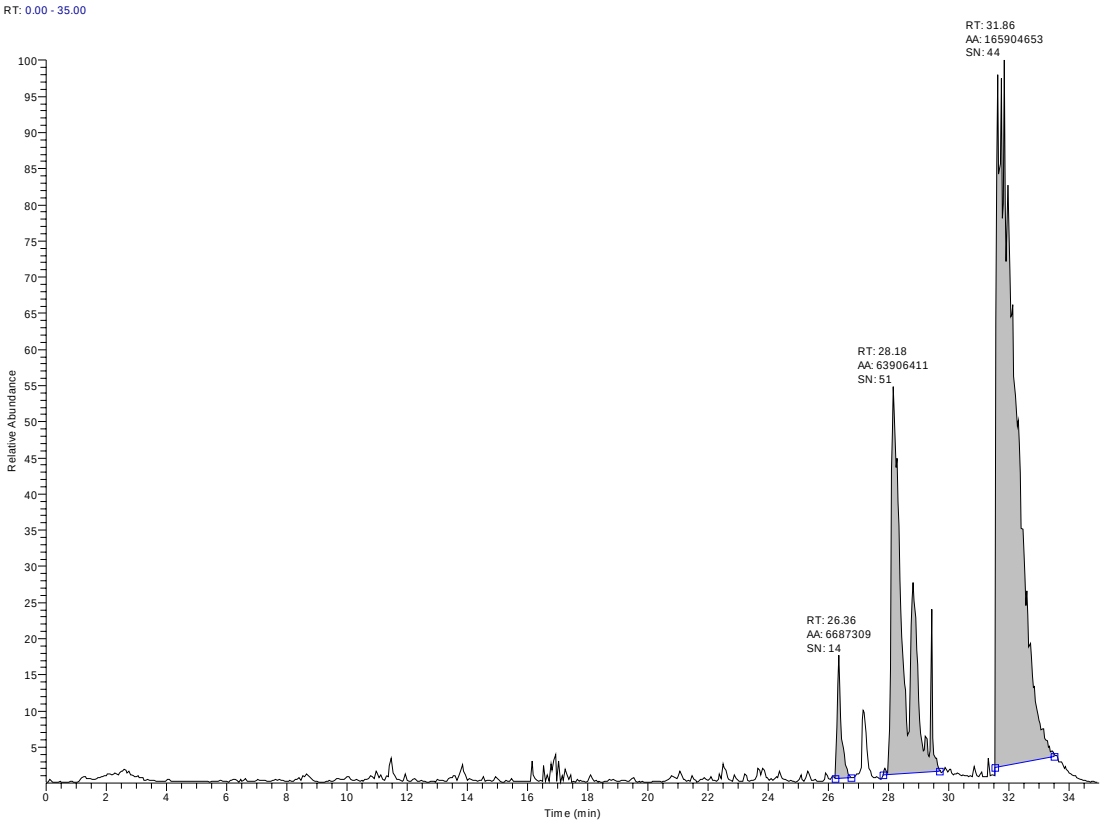
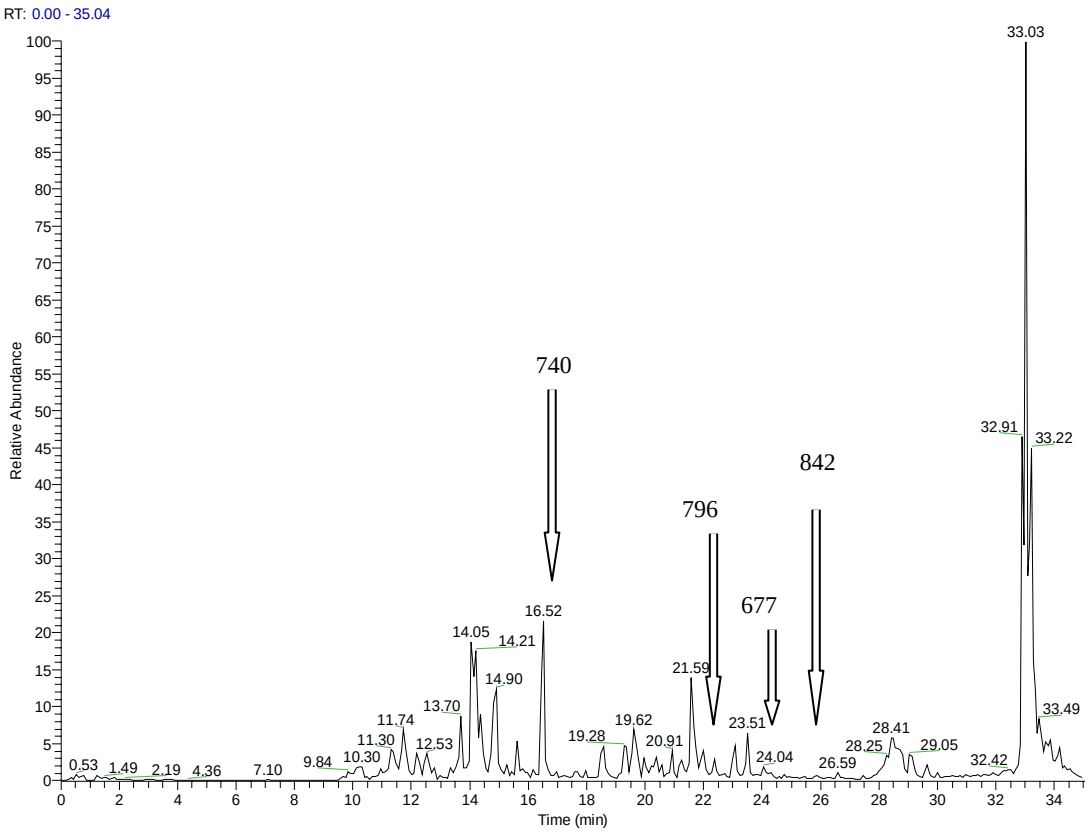



**Fig. 18: Chromatogram of water phase after the separation (typical for all the separations)**



The most important thing, we can see on these chromatograms is a remarkable difference of lipophility between farnesylated (796,677,842) and non-farnesylated peptides (740). Most expressive is this between 740 and 842, which represent the same peptides, only 842 is farnesylated. The arrows indicate peptides: 740 – LGEYGFQNALIVR

796 - YIFar-CDNQDTISSK

677 - RPFar-CFSALTPDETYVPK

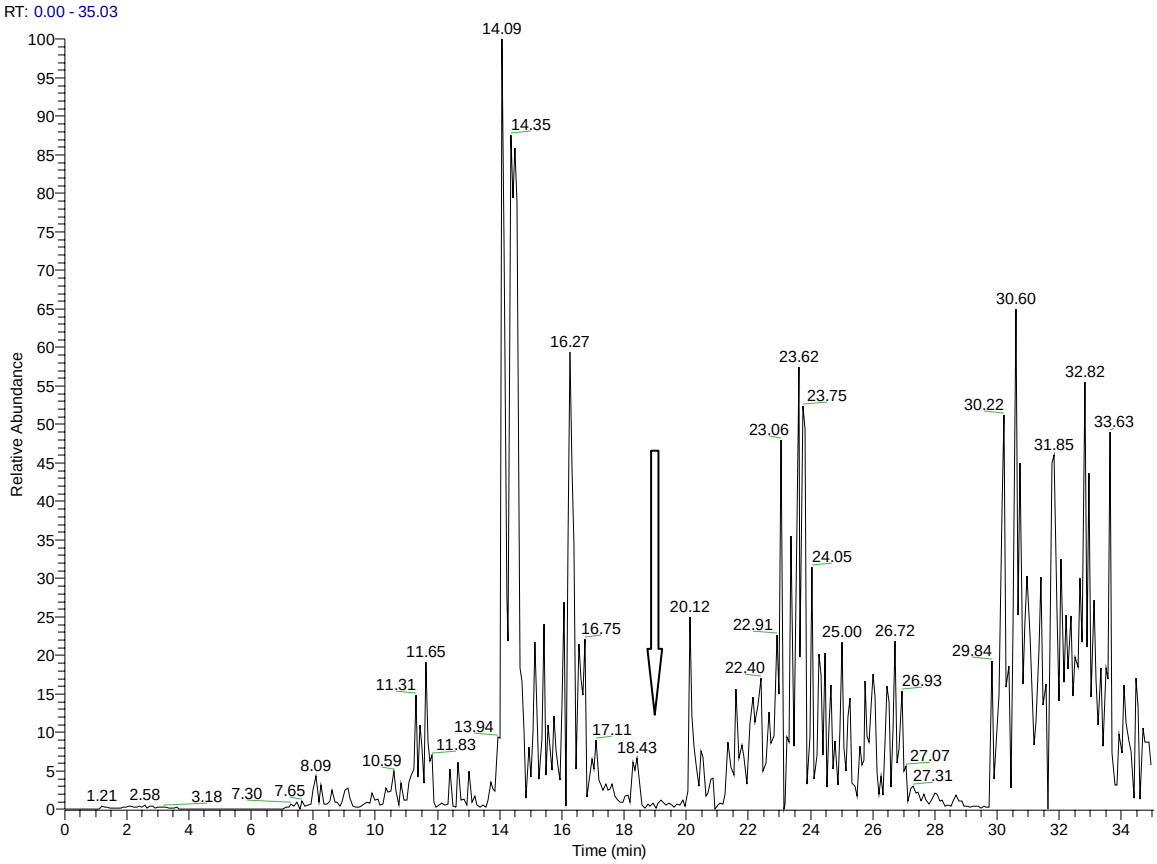
842 – Far-LGEYGFQNALIVR

From these methods of separation, probably the normal Folch method was the best one, because most of the interesting peptides were found there, whereas in the isopropanol/chloroform/water method I had a bit of a trouble finding the peptides and the ethyl acetate/water method did not separate peptides at all, just lipophilic impurities. Also compared to the i/c/w method, Folch is much simpler to carry out. The main advantage of the separation process is that the spectrum is afterwards somewhat simpler as not all the peptides are still in the sample. However distinction on farnesylated and non-farnesylated peptides by these methods is not complete and other method should be developed to do so.

## MCF-7 spectra interpretation

The chromatogram of the sample does not contain any vital information for the identification of proteins, we can just note a big amount of unclear peaks. An arrow indicates the peptide RNDALPTSLFar-CLCFar-CSEN (see further) (Fig. 19).

**Fig. 19: Chromatogram of MCF 7 cells digested membrane proteins sample**




The MS data were analyzed by Bioworks browser with the search adjusted to find modified cysteine by farnesyl, methyl and also palmitoyl. A number of different farnesylated/palmitoylated peptides were found by the programme, but none of them was characterized by more than one peptide fragment or more than 15 % match with the total mass of the peptide. These peptides included also non–human peptides like the protein phosphatase from Caenorhabditis elegans roundworm, which was characterized by a long fragment with 4.7 % match. Therefore an extra selection was necessary to pick relevant results.

In the end only a couple of human proteins remained after the selection. Two of them were represented by the same peptide ion fragment, a triple-charged 1144 peak. Even after the MS/MS analysis of this peak both peptides were characterized by different fragments from the same spectrum of the parent ion. One of them should have been a farnesylated peptide from geranylgeranyl pyrophosphate synthase –

TEMVAGQYLDLRGQLSGGSSVAQALRTAFar-CLK with a 10.2 % match to the protein. The other one should have been from a regulating autophagy protein – palmitoylated and farnesylated peptide QRVSYPTAEuFar-CCQHLGILvPal-CLCSRCSGTR with a match of only 2.5 % to the protein mass. After a closer analysis of the spectrum, I have come to a conclusion, that it is neither of the two previous peptides. This only illustrates how demanding and tricky it is to analyze and recognize proteins from a complex sample made of cells.

The most promising was a peptide fragment, which was a terminal part of human uncharacterized protein product with a match of 11 % by mass. The fragment ion is double-charged 1075 and stands for a double farnesylated RNDALPTSLFar-CLCFar-CSEN (see Fig 20). The MS/MS analysis of this parent ion gives us new peaks that characterize the peptide, main peaks can be explained as follows: 349 y-ion SEN; 553 b-ion RNDAL with a loss of molecule of ammonia; 571 b-ion RNDAL; 892 double-charged b-ion RNDALPTSLFar-CLCFar-C with a loss of molecule of water; 935 double-charged b-ion RNDALPTSLFar-CLCFar-CS with a loss of molecule of water; 1294 y-ion LFar-CLCFar-CSEN; 1381 y-ion SLFar-CLCFar-CSEN; 1561 y-ion PTSLFar-CLCFar-CSEN with a loss of molecule of water; 1579 y-ion PTSLFar-CLCFar-CSEN; 1692 y-ion LPTSLFar-CLCFar-CSEN; 1801 b-ion RNDALPTSLFar-CLCFar-C; 1888 b-ion RNDALPTSLFar-CLCFar-CS (see Fig 21). The farnesylation in this peptide is however unlikely, because the typical peaks of the farnesylated peptides as were seen before are not in the spectrum. These typical peaks should be the peptides with cleaved-off farnesyl. In this case a peak of 974 (still with one farnesyl) or 873 (with both farnesyls gone) would be expected.

**Fig. 20: Full MS spectrum of RNDALPTSLFar-CLCFar-CSEN peptide fragment from uncharacterized protein product**

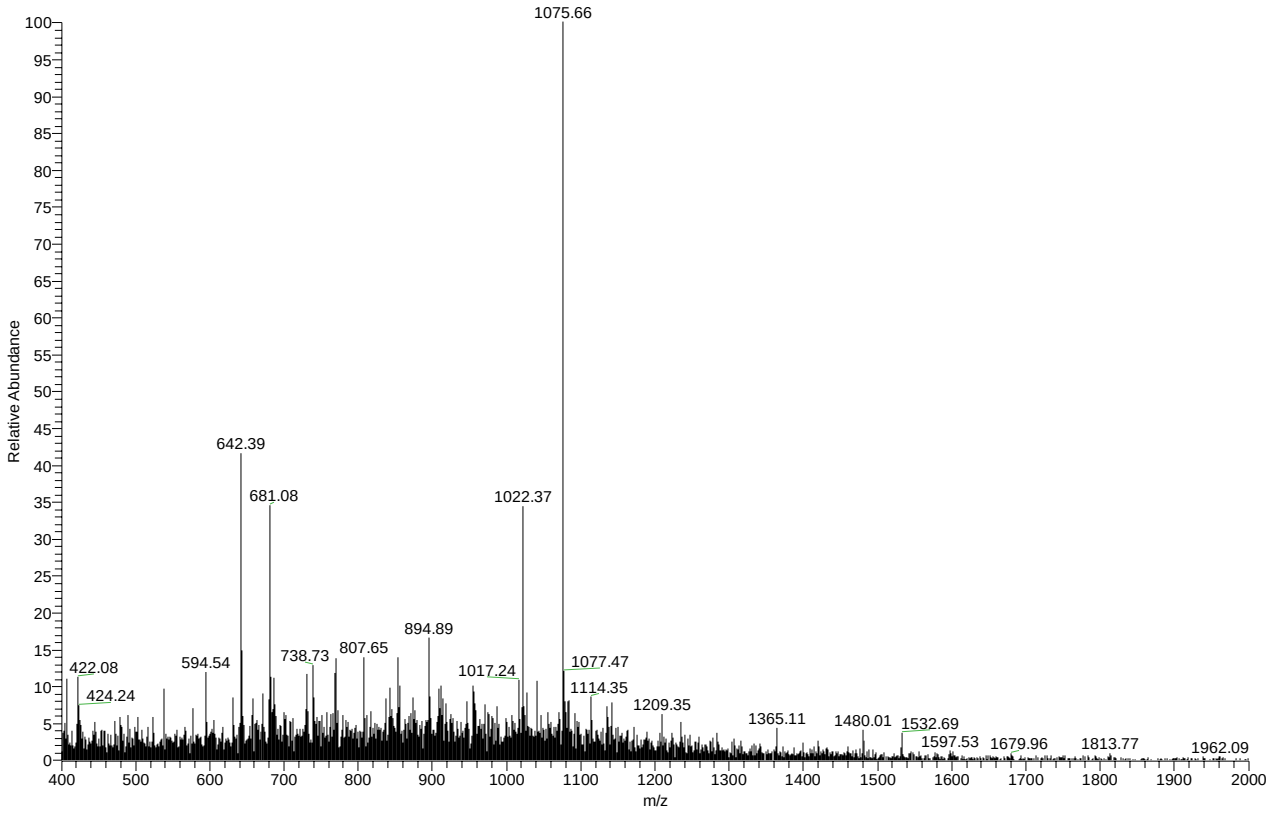
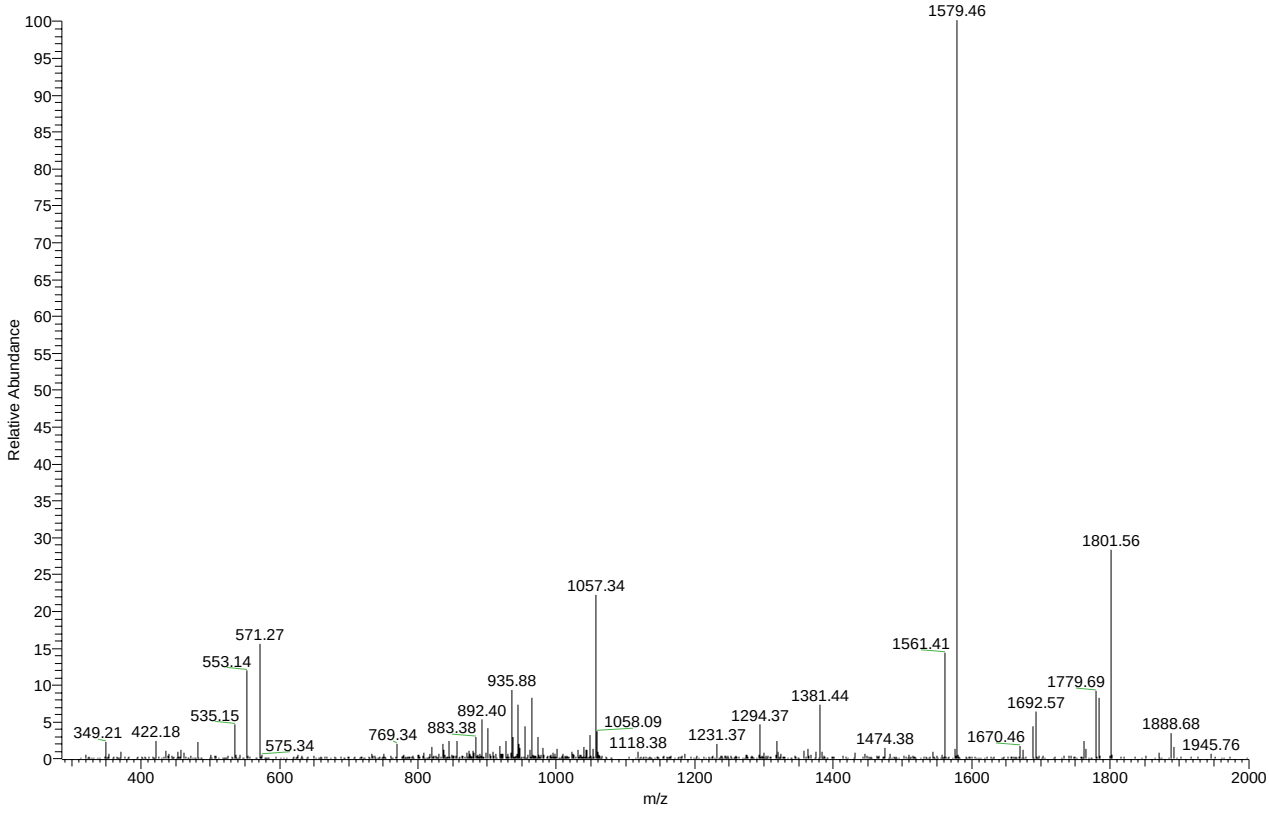



**Fig. 21: MS/MS spectrum of a RNDALPTSLFar-CLCFar-CSEN peptide (parent ion 1075)**



The observed outcome of the analysis proved that there were no peptides from the plasma membrane, which were desired, or they were in an undetectable amount (the boundary concentration value is 100pM!). The method of the farnesylated proteins detection by MS is clear, all that would be needed to see them is to isolate them in proper amount to the sample. Therefore it is necessary to further adjust the method of their separation from the rest of the cell ballast.

# Conclusion

The farnesylated peptides have been successfully characterized from the two simple peptides and mainly from farnesylated peptides derived from albumin. The cleavage of these peptides, when subjected to MS/MS analysis, was determined so that farnesylation can be identified very reliably in every protein sample, that contains the proteins enough concentrated. We cannot see any peak for farnesyl itself, but the spectra of farnesylated peptides show at least two typical peaks. Both result from the detachment of the farnesyl moiety from the peptide itself, but each of them is created in a different manner. One is a peak representing double-charged fragment originated by homolytic cleavage, therefore leaving another charge on the peptide fragment. The other peak is a single-charged fragment formed by a loss of neutral farnesyl moiety, leaving no additional charge on the peptide. With these simple rules, I am sure that every farnesylated protein and its resulting peptides can be viewed and recognized using electrospray mass spectrometer.

The search for farnesylated peptides in the protein sample, made by isolation of cell membrane proteins from breast cancer cells, was a little bit disappointing. There was no direct evidence of farnesylated proteins at all. This was definitely caused by a too low concentration of these proteins in the sample. The method of isolation, which I used in my work, was obviously not good enough to isolate a distinguishable amount of proteins from a relatively high quantity of biological material. The separation technique, which was tested on the peptides from farnesylated albumin to give a better resolution and view of farnesylated peptides, could not aid us in providing higher yields of farnesylated proteins from cells. Because of the lack of time, I was forced to abandon the pursuit for a simple method development, by which farnesylated proteins obtained from cells would be detected. Next step would be to continue the search for a process, how to obtain cell membrane

proteins, but maybe even complete cell proteins, in a sufficient quantity for the MS analysis. However, that will be a task for some next consequent work.

# Supplements

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# Abstract

Farnesylated proteins are important in transduction of signals in cell and therefore can figure in development of many diseases, mainly cancer. Electrospray mass spectrometry is a widely used method in analysis of peptides and proteins, thus it was chosen as a method to develop a simple way to detect farnesylated proteins in cell. A cleavage pattern of these proteins, when subjected to MS/MS, was found on examples of synthetically farnesylated simple peptides and bovine albumin. Distinctive features of MS/MS spectra of these peptides are two peaks, which both represent the virgin peptide fragment after the farnesyl moiety was cleaved off. These fragments have a different charge, because they originate from different type of cleavage. Homological cleavage of the bond between the farnesyl and sulfur leads to formation of a new charge on the peptide fragment, while the leaving farnesyl is also charged and has amu of 205. Second type of fragment rises from neutral loss of farnesyl moiety, there is no new charge generated on the peptide fragment and the amu of the leaving farnesyl moiety is 204, because it looses one of its hydrogen in favor of the peptide. This knowledge can be applied also to more complicated protein samples prepared from cells. When searching for farnesylated proteins in such a sample made from cell membrane proteins of breast cancer cells a new problem occurred and greatly complicated the determination – the proteins were in such a low concentration that it was immensurable. Therefore a method to obtain these proteins from cells in a satisfactory concentration has to be developed.

# Krátké shrnutí práce

Farnesylované proteiny mají velký význam ve vedení signálu buňkou a jako takové mohou hrát roli ve vzniku řady chorob, zejména rakoviny. Hmotnostní spektrometrie s ionizací elektrosprejem je metoda široce využívaná v analýze peptidů a proteinů, proto byla zvolena k vyvinutí snadného způsobu nalezení farnesylovaných proteinů v buňce. Na synteticky farnesylovaných jednoduchých peptidech a hovězím albuminu byl objasněn způsob, jakým se farnesylované peptidy štěpí v hmotnostním spektrometru. Charakteristickým rysem MS/MS spekter těchto peptidů jsou dva píky, které vznikly odštěpením farnesylu od samotného peptidu. Tyto fragmenty se liší v počtu nábojů na fragmentu, to je způsobeno rozdílným druhem štěpení. Rozštěpením vazby mezi sírou cysteinu a farnesylem rovnoměrně, vzniká nový náboj na peptidu a odstupující nabitá skupina o atomové hmotnosti 205. Pokud štěpení probíhá za ztráty neutrálního fragmentu farnesylu, na peptidu zůstává pouze původní náboj a odstupuje fragment o atomové hmotnosti 204, který ztratil jeden atom vodíku ve prospěch peptidu. Tato zjištění lze aplikovat z jednoduchých peptidů i na hledání farnesylace ve vzorcích, obsahující složitou směs proteinů z buněk. Při hledání farnesylovaných proteinů ve vzorku získaném z proteinů buněčné membrány buněk rakoviny prsu, jsem však narazil na problém neměřitelně nízké koncentrace farnesylovaných proteinů. Musí tedy být vyvinuta metoda jejich izolace z buněk v dostatečném množství.