Investigation of the reactivity of the skin allergen limonene-1-hydroperoxide with amino acids and identification of adducts with LC/ESI-MS/MS.

Diploma thesis

STOCKHOLMS UNIVERSITET
DEPARTMENT OF ANALYTICAL CHEMISTRY

UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
KATEDRA ANALYTICKÉ CHEMIE

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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla využita k získání jiného nebo stejného titulu.“

V Hradci Králové 2011 Karolina Škrášková

Nejčastější příčinou toho, že nedosáhneme svých cílů je fakt, že zabíjíme čas tím, že děláme nejdříve druhořadé věci.

Robert J. McKain

All paid jobs absorb and degrade the mind.

Aristotle

Contents

Abbreviations

ACD	Allergic Contact Dermatitis
ACN	Acetonitrile
Ala	Alanine
API	Atmospheric pressure ionization
CAD or CID	Collision Activated / Induced Dissociation
Cys	Cysteine
DC	Direct current voltage
ESI	Electrospray ionozation
Fig.	Figure
His	Histidine
HPLC	High-performance liquid chromatography
IT	Ion trap
Leu	Leucine
Lim-1-OOH	Limonene-1-hydroperoxide
Lim-2-OOH	Limonene-2-hydroperoxide
LLNA	Local lypmh node assay
Lys	Lysine
m/z	Mass-to-charge ratio
MALDI	Matrix assisted laser desorption/ionization
MS	Mass Spectrometry
Q	Quadrupole
QqQ	Triple Quadrupole
Ref.	Reference
RF	Radiofrequency
RIC	Extracted Ion Current
RT	Retention time
SRM	Selected reaction monitoring
Tab.	Table
TIC	Total Ion Current
TMIO	1,1,3,3-tetramethylisoindolin-2-yloxyl
Trp	Tryptophane
Tyr	Tyrosine

Abstrakt

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Název diplomové práce: Investigation of the reactivity of the skin allergen limonene-1-hydroperoxide with amino acids and identification of adducts with LC/ESI-MS/MS.

Kontaktní alergie a její klinický projev alergická kontaktní dermatitida představují vážný zdravotní problém vyspělých zemí. Jedná se o zánět kůže projevující se jako vyrážka (ekzém). Již u více než tří tisíc chemikálií byl prokázán alergenní potenciál.

Limonen je monoterpen běžně používaný jako vonná přísada v čistících prostředcích a kosmetických výrobcích. Limonen není alergenní sloučenina, ale na vzduchu vytváří směs oxidačních produktů, které jsou více či méně silnými alergeny. Z této směsi byly hydroperoxidy limonenu identifikovány jako sloučeniny se silným alergenním potenciálem. Ze směsi oxidačních produktů byly jako dvě z hlavních sloučenin izolovány limonen-1-hydroperoxid a limonen-2-hydroperoxid.

Výzkum kontaktní alergie probíhá již několik desetiletí. Během této doby bylo prokázáno, že alergenní sloučeniny reagují s proteiny kůže. Tato reakce spouští kaskádu komplikovaných imunologických procesů, na jejichž konci stojí zánětlivé onemocnění kůže. Většina chemikálií vytváří vazbu s proteiny prostřednictvím elektrofilních a nukleofilních interakcí. Odhaduje se však, že právě hydroperoxidy tvoří komplexy s kožními proteiny radikálovým mechanismem.

Reaktivita obou hydroperoxidů limonenu byla již zkoumána v několika projektech. V jednom z nich byly určovány struktury komplexů mezi limonen-2-hydroperoxidem a vybranými aminokyselinami.

Následující projekt je zaměřen na reaktivitu druhého z hydroperoxidů limonenu. Zkoumána je tvorba komplexů mezi limonen-1-hydroperoxidem a aminokyselinami. Cílem je i určení nebo alespoň odhadnutí struktur vzniklých aduktů s využitím LC/ESI-MS/MS.

Výsledky výzkumu jsou nezbytné pro komplexní pochopení mechanismu kontaktní alergie a pro ohodnocení alergického potenciálu dalších sloučenin.

Abstract

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Diploma Thesis Title: Investigation of the reactivity of the skin allergen limonene-1-hydroperoxide with amino acids and identification of adducts with LC/ESI-MS/MS.

Contact allergy and its clinical manifestation allergic contact dermatitis represent a serious problem in western countries. Thousands of chemicals have been proven to cause skin sensitization followed usually by an inflammation of the skin.

Limonene is a monoterpene used as a fragrance compound in household products and cosmetics. It is itself a non-allergenic compound. Nevertheless after an air exposure it creates oxidized products, which are potent allergens. The hydroperoxides fraction of the oxidation mixture has been shown to be strongly skin sensitizing. Two of the major compounds in the fraction are limonene-1-hydroperoxide and limonene-2-hydroperoxide.

During decades of research devoted to the contact allergy it was revealed, that the chemicals causing allergic contact dermatitis react with skin proteins, thus starting up a complicated cascade of immunological processes leading to the final skin irritation. The reaction between the chemical and the skin protein is usually based on electrophilic-nucleophilic interactions. The hydroperoxides are however believed to react via radical mechanism.

In several projects the reactivity of the limonene-hydroperoxides was examined. In amino acid-based assays adducts between limonene-2-hydroperoxide and particular amino acids were investigated and structures of adducts were determined.

In this project we follow the research on adducts with limonene-2-hydroperoxide, but focusing this time on limonene-1-hydroperoxide. The formation of adducts is examined and their structures are suggested and/or determined using LC/ESI-MS/MS.

The research on adduct formation with limonene hydroperoxides is crucial for understanding the mechanism of contact allergy towards this type of compounds and for evaluating their allergenic potential.

Aim of the thesis and assignement of the work

The project follows the research of the reactivity of limonene-2-hydroperoxide with amino acids (*ref.1*). Limonene-1-hydroperoxide was found to be stronger skin sensitizer from the two limonene hydroperoxides. In this thesis the reactivity of limonene-1-hydroperoxide is therefore aimed. The intention was to reveal, whether limonene-1-hydroperoxide reacts in the amino acid models assay in the same fashion as limonene-2-hydroperoxide and suggest the structures of the estimated adducts. The project involved three steps: screening experiments, control samples experiments and MS^n experiments.

PART 1 - Theory

1. Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy [1]. It manifests itself as a skin irritation or eczema. The skin reaction is caused by a prolonged or repeated exposure to certain chemicals or metal ions [2].

In the process of ACD the immune system is crucial. ACD is a hypersensitivity reaction of IV type, which is also called delayed or cell mediated. Delayed refers to time, which the irritation occurs in after the exposure. It takes usually between 24 and 72 hours [3]. Cell mediated refers to T-cells, which intermediate the immunogenic reaction.

ACD proceeds in two phases: the first one is called *sensitization ph*ase and the second *elicitation phase* [3]. The sensitization phase occurs during the first exposure to the allergen. The allergens are also called haptens. Haptens are in general low-molecular (<1000 Da) compounds lipophilic enough (log P ~2) to penetrate the skin. Nevertheless haptens themselves are too small molecules to elicit an allergic response after the skin penetration. Thus the second requirement of the elicitation of an immune response is the reaction of hapten with the skin proteins [2].

The structures created from the hapten and skin proteins are called immunogenic complexes. These complexes are processed by Langerhan's cells (LCs). LCs are the main antigen-presenting cells in the epidermis. They become activated once the proteins are haptenated. LCs process the formed immunogenic complexes and represent them to naive T-cells as an antigen. Such interactions lead to a formation of antigen-specific memory T-cells, which are thereafter present in the blood circulation [2].

The elicitation phase is caused by a renewed contact with the allergen. The memory T-cells, which are now present in the blood, are activated by the formation of the specific antigen. Activation of the memory T-cells leads to a formation of the effector T-cells. These cause a releasing of the pro-inflammatory substances causing an inflammation on the site of the skin contact [1, 3]. The dermatitis (eczema) appears, when a sensitized person is exposed to an allergen in a concentration high enough [2].

ACD represents an increasing health problem of modern society. In western countries about 15-20 % of population suffers from ACD [2]. ACD is regarded to be a chronic disease. This means, that once the problem appears, it is very difficult, almost impossible to eliminate it. The only solution is to avoid contact with the allergen [3]. Corticosteroids represent the only available symptomatic treatment. Some investigations have been led also in the field of the specific treatment [4].

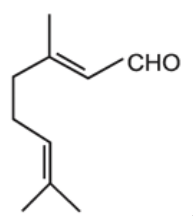
The ACD research is in general spread in two main fields: recognition of the process of protein haptenation and discovering the exact mechanism of activation process of the non-allergenic compounds (prohaptens) either by air or metabolically (*see chapter 2.2.*). The results are important for i) faster and more accurate determining of new potential allergens, ii) development of in vitro testing methods of the allergens [5] and iii) down-regulation of use of the allergenic compounds in everyday-used products.

2. Allergens

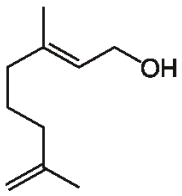
The most common allergens causing ACD are metal ions (nickel, chromium, cobalt) and some aldehydes and terpenes used typically as fragrance compounds or present in turpentine and colophony (Δ^3-carene, abietic acid). So far more than 3 000 chemicals have been shown to cause skin sensitization resulting in ACD [2].

Fragrance compounds are used in various detergent and cosmetic products to provide more pleasant (even though artificial) scent. Typically aldehydes and/or terpenes are used. Some of them are shown in Fig.1.

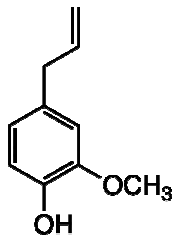
Fig.1. Common fragrance compounds



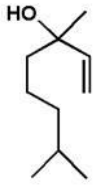
Citral



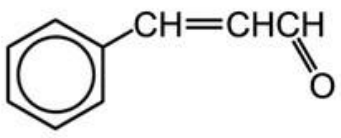
Geraniol



Eugenol

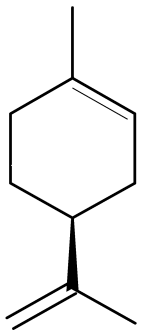


Linalool



Cinnamal

2.1. Limonene



R-(+)-Limonene (d-limonene) is a monoterpene naturally occurring in oil of fruit genus *Citrus*, in caraway, dill and celery [6]. It is one of the most commonly used fragrance chemicals in scented products [7]. Limonene is usually used as a flavouring ingredient in domestic products and perfumes in concentration below 0.03%. Due to its solvent capacity it is added to cleaning products in a concentration of several %. Recently limonene has been used in industry to replace more toxic substances such as chlorofluorocarbons. In some histological laboratories it has been used as a replacement for xylene [6]. In therapeutic transdermal delivery systems it is used as a penetration enhancer [8]. Limonene itself is a non-allergenic compound. Nevertheless many cases of ACD related to limonene have been reported [9].

2.2. Activation

Limonene is an example of a prohapten. Prohaptens are in general compounds, which sensitize the skin after they had been activated i.e. after their structure had been modified. Knowledge about the process of activation is unfortunately limited. The process itself is usually caused either by oxidation of the chemical on air or metabolically by enzymes present in the skin. The influence of enzymes of cytochrome P 450 expressed in the skin on activation of prohaptens was investigated in *ref. 10*. (Other sources distinguish between the words *prohapten* and *prehapten*: saving the prohapten for the chemicals activated enzymatically and prehapten being the chemicals activated in different ways [11].) In *ref. 12* some of the conjugated dienes have been shown to act as prohaptens in ACD.

Limonene as a monoterpene with double bonds is prone to self-oxidation. Oxidative degradation of limonene was shown to occur at room temperature [7]. The sensitizing potential of limonene increases with the prolonged air-exposure [6]. Investigation of limonene air-exposed samples by gas chromatography revealed composition of the samples: limonene, limonene-1,2-oxide, carvone, carveol and limonene-hydroperoxides, whereas content of limonene was obviously lowered [13].

Ability of non-oxidants to stop the oxidation process was investigated in *ref. 14*. In *ref. 15* self-oxidation of linalool was examined and herein obtained results correspond with limonene.

2.3. Hydroperoxides

The limonene-hydroperoxides were confirmed to be potent contact allergens in guinea pigs studies [13]. When patch-testing in human dermatitis patients was accomplished, the limonene-hydroperoxides have been considered to be the most important allergens of the limonene oxidation mixture [16].

Limonene-1-hydroperoxide and limonene-2-hydroperoxide are the two of the hydroperoxides naturally occurring in oxidized limonene. Sensitizing capacity of mentioned hydroperoxides was investigated in local lymph node assay. Both of them were discovered to be strong sensitizers, whereas limonene-1-hydroperoxide showed lower EC3 values and thus was confirmed to be more potent sensitizer from the two hydroperoxides (*see chapter 5.3.*) [3, 11].

Potential cross-reactivity of the hydroperoxides was investigated. Cross-reactivity is caused by creating non-specific antigens. If two chemicals cross-react, a person sensitized by one of them will show symptoms of ACD also after exposure to the second chemical. However, cross-reactivity between limonene-hydroperoxides was not confirmed, stating that limonene-hydroperoxides create specific antigens in ACD [17].

3. Reactivity of allergens

The crucial process for outburst of ACD is the formation of immunogenic complexes (antigens), i.e. haptenation of the skin proteins with allergens (haptens). The haptenation is determined by the physical-chemical properties of the hapten (size of the molecule and lipholicity) [2] and by its reactivity. Thus only the protein-reactive chemicals (or those, which can be activated – s*ee chapter 2.2.*) have the potential to act as skin sensitizers [18]. The bond which is created between the hapten and the skin proteins is usually either covalent or coordination [3].

3.1. Electrophilic-nucleophilic interactions

The majority of sensitizing chemicals are electrophiles. On the other hand the amino acids are rich in nucleophilic side chains, thus being an ideal target for the small electron deficient molecules of haptens. The strongest nucleophilic targets in proteins are N-terminal amino groups of amino acids in general, cysteine sulfhydryl group, histidine imidazole group and

lysine ε-amino group [18]. Lysine and cysteine are the nucleophilic targets most often cited, but other amino acids containing nucleophilic heteroatoms, for example histidine, methionine, and tyrosine, can also react with electrophiles [19]. Important are also the values of pH in the skin [18].

About 2 000 different proteins in the skin have been so far identified. The chemicals usually bind to a broad range of the proteins without specific targeting of a particular protein [18].

3.2. Radical mechanism

At the beginning of 1990' contact allergy to rosin (colophony) was investigated. The main allergen in rosin has been identified as abietic acid or rather its oxidated product: 15-hydroperoxyabietic acid. Further investigation of the possible routes of protein haptenation followed [20]. *Ref. 21* shows results of research regarding the interaction of synthesized allylic hydroperoxides with proteins.

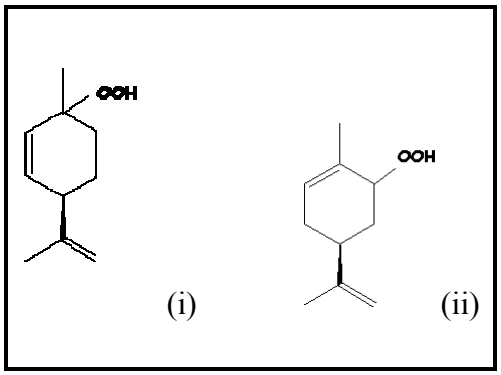
In so far mentioned papers was postulated that hydroperoxides react with proteins in a radical pathway rather than by electrofilic-nucleophilic interactions. This statement has been later developed in other studies concerning the radical mechanism of haptenation by hydroperoxides [1, 3, 19, 22, 23, 24, 25]. Compounds investigated in *ref. 20 and 21* are in narrow correlation to limonene due to the same structure of limonene oxidation products. Therefore it is proposed that the radical mechanism is preferred also by limonene hydroperoxides during the haptenation of proteins in ACD process.

The radicals formed from hydroperoxides in the skin increase the oxidative stress by consumption of the antioxidants. The antioxidants are present in the skin to balance the level of radicals, which are constantly formed in our cells. However, the increased oxidative stress evokes the radical formation of macromolecules such as proteins and lipids. Thus the weakened antioxidant defence of the skin facilitates the haptenation of skin proteins by products formed from hydroperoxides via radical mechanism [1, 3].

Because hydroperoxides are oxidizing agents, they could in theory form non-specific antigens by oxidation of skin proteins. Nevertheless cross-reactions between the hydroperoxides would be observed, as structurally different hydroperoxides would form the same oxidated proteins. However, there is no support for such a theory in the literature and recent studies have not confirmed it [1].

3.3. Limonene hydroperoxides radicals

Fig.2. Limonene hydroperoxides



Limonene creates a mixture of oxidation products on air-exposure. Among the oxidation products two hydroperoxides have been shown to be strong skin sensitizers (Fig.2.): limonene-1-hydroperoxide (4R)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide) (i) and limonene-2-hydroperoxide ((5R)-5-isopropenyl-2-methyl-2-cyclohexene-hydroperoxide) (ii) [3].

Hydroperoxides are metabolized by cytochrome P450 enzymes. Cytochrome P450 represents a large family of enzymes, which are present mainly in liver, but also in skin and other tissues [3, 10]. The P450 causes the homolytical cleavage of oxygen-oxygen bond of the hydroperoxides [3, 24]. It was revealed that the main active part of P450 is the heme containing unit [3].

When the radical formation of the hydroperoxides was studied, it was necessary to use a radical inducer which would biomimetic the homolytical cleavage of oxygen-oxygen bond. An inducer which is a model of the heme group in P450 was found in Iron (III) porphyrin complex (5,10,15,20-tetraphenyl-21H,23H-porphine iron(III) chloride, [Fe(III)TPPCl3]) [3].

Mechanism of O-O bond cleavage in hydroperoxides by Iron (III) Porphyrin complexes was in detail examined in *ref. 22*. According to the results the cleavage of the O-O bond may be either homolytical or heterolytical in dependence on i) electron nature of the used complex and ii) on substituents of the hydroperoxide.

For the investigation of limonene hydroperoxides radical formation electron paramagnetic resonance and radical trapping experiments were performed. Two elementary radicals formed from hydroperoxides were revealed: oxygen-(O-) centered and carbon-(C-) centered radicals. Both carbon- and oxygen-centered radicals are important in the formation of immunogenic hapten-protein complexes of hydroperoxides [24, 25]. Nevertheless it was implied O-centered radicals being of higher importance regarding the creation of immunogenic complexes [25].

Oxygen-centered alkoxy radicals are formed by breaking the oxygen-oxygen bond. The formed oxygen-centered radicals could be than rearranged into other radical or nonradical species, which can also act as haptens [24]. Three major pathways of alkoxyl radicals transformations were revealed: *hydrogen abstraction* (resulting in analogous alcohol),

1,2-shift (generating carbon-centered R-hydroxyl radical) and *1,3-cyclization* (yielding the creation of primary or secondary carbon-centered alkyl radicals.) The structure of the hydroperoxides is meanwhile determining for the route of the radical formation *(see chapter 5.1.)* [25].

Ability of the studied hydroperoxides to form peroxyl, allyloxyl, and oxiranylcarbinyl radicals has been proven in *ref. 24*. It was further stated that these radicals can be involved in antigen formation in ACD process [24]. Although unspecific oxidation of proteins also occurs in the presence of radicals derived from hydroperoxides, there is no evidence for the formation of specific immunogenic complexes via this mechanism [25]. Alternatively compounds acting as electrophiles could be formed from hydroperoxides by intramolecular radical rearrangements. Such compounds would haptenate the proteins in classical electrophilic-nucleophilic mechanism [1].

The cited statements regarding the structure of radicals formed from hydroperoxides can be further supported by investigation of linalyl hydroperoxide in other experiments. The results of the research also confirmed the creation of oxygen- and carbon-centered radicals [19, 23].

4. Mass Spectrometry [26 – 31]

In whichever book about mass spectrometry (MS) the authors usually do not forget to mention that MS has passed through a fast and tremendous progress in past century. The very first mass spectrometer was constructed by Sir J.J.Thomson at the beginning of 20th century [26]. Since that time the analytical technique has been developed and improved in all its matters.

The main application of MS is determination of the structure of numerous compounds. The essence of the quite complicated apparature is in a production of gas phase ions of particular compounds. The created ions are later separated according to their mass-to-charge ratio (m/z).

The four basic components of each mass spectrometer are sample inlet, ionization source, mass analyzer and detector. Computer software is an integral part of the whole determination process. The pumps provide a very low pressure, which is necessary for the transport of ions into the mass spectrometer.

4.1. Sample introduction

Sample introduction would be expected to meet the problem with pressure difference. The sample is inserted under the atmospheric pressure to a relative vacuum of the mass spectrometer. Currently there are two different types of sample introduction each of them smartly solving the presented problem: i) direct insertion using an insert probe or plate is commonly used with MALDI (Matrix Assisted Laser Desorption/Ionization), ii) direct infusion or injection is conveniently used with ESI (Electrospray Ionization).

4.2. Ionization methods

The term is often confused with ionization sources. Meanwhile ionization sources are mechanical devices, the ionization methods regards the mechanism of the ionization, i.e. transfer of the compounds to the ions. The available ionization methods are: protonation, deprotonation, electron ejection, electron capture, cationization, anionization and transfer of a charged molecule to gas phase [26].

4.3. Ionization sources and ionization methods

As the structure of the compound can be determined only in the state of ions, the ionization itself is a crucial step in the whole analysis. The ionization source converts the neutral compound into the ions. The mechanism of converting is called ionization method *(see chapter 4.2.)*.

The ionization sources may be divided into two groups according to the amount of energy inserted to the compound. So called soft MS methods produce only ions of the molecular species, meanwhile hard methods cause extensive fragmentation of the compound [27]. Electron ionization is an example of a hard method, while chemical ionization, thermospray and electrospray ionization are classified as soft.

The originally invented ionization methods could be used only for determination of low-molecular compounds. As the time passed by, new mechanical devices and new principles have been developed. Now the structure of macromolecules such as proteins, synthetic polymers or polynucleotides can be determined. For that reason, some of the main fields of MS application now comprise biotechnology and biology systems.

4.3.1. Electrospray ionization [32, 33]

Chapman performed the first experiments on electrospray ionization (ESI) in the late 1930's. It was Dole, who shifted forward the development of ESI three decades later. And lately Fenn, whose work led to a current modern technique [26], was awarded a shared Nobel Prize for Chemistry in 2002 for further developing the ESI method [32].

The original intention for ESI application was aimed to protein analysis. It was later, that its use has been enriched by the analysis of other polymers and biopolymers and also small polar molecules [27]. Nowadays it is routinely used with peptides, proteins, carbohydrates, small oligonucleotides, synthetic polymers and lipids [26].

Due to an ESI development, the analysis of proteins and nucleic acids with molecular masses higher than 100 000 Dalton has been enabled. Moreover it shows to be a powerful tool in determining the structure of antigen-antibody complexes [33].

However the main advantages of this ionization technique remain the high sensitivity and possibility of straight-forward coupling with high-performance liquid chromatography (HPLC). Together with MALDI is ESI the most frequently used ion source for coupling LC/MS.

4.3.1.1. Principle

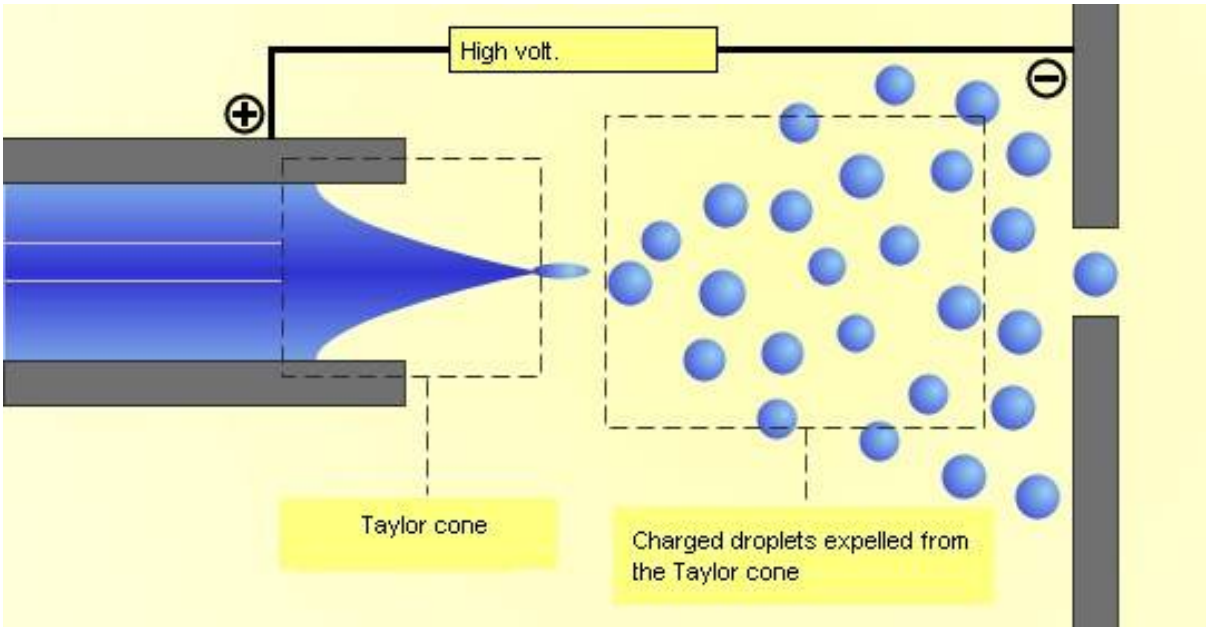
The ESI operates under an atmospheric pressure and is also referred to as one of the atmospheric pressure ionization (API) techniques [29]. It represents a soft-ionization technique based on desorption of ions from liquid to gas phase [33].

Its principle is consisted of following processes: nebulization, desolvation, ionization, transportation and later detection in the mass spectrometer [32]. The whole ESI process might be divided in three fundamental steps: i) nebulization of analyte solution to a fine aerosol containing small charged droplets, ii) desolvation of ions from the surface of charged droplets, iii) transfer of ions from a state under the atmospheric pressure to the vacuum of mass analyzer.

The base mechanism of ESI is provided by a strong electric field. High voltage is applied on a capillary with the analyte solution and opposite standing counter electrode. The potential difference is usually 2-5 kV. Due to the voltage applied to the capillary (usually being positive) a separation of charges inside the capillary occurs. The electric field makes the

liquid being sputtered from the capillary in the shape of a cone, also called as the "Taylor cone". When the surface tension and electrostatic repulsion are equal (the Rayleigh limit is reached), the charged droplets are expelled from the tip of the cone as it s depicted in Fig.3. [26, 32].

Fig.3. ESI: Taylor cone and forming of the droplets



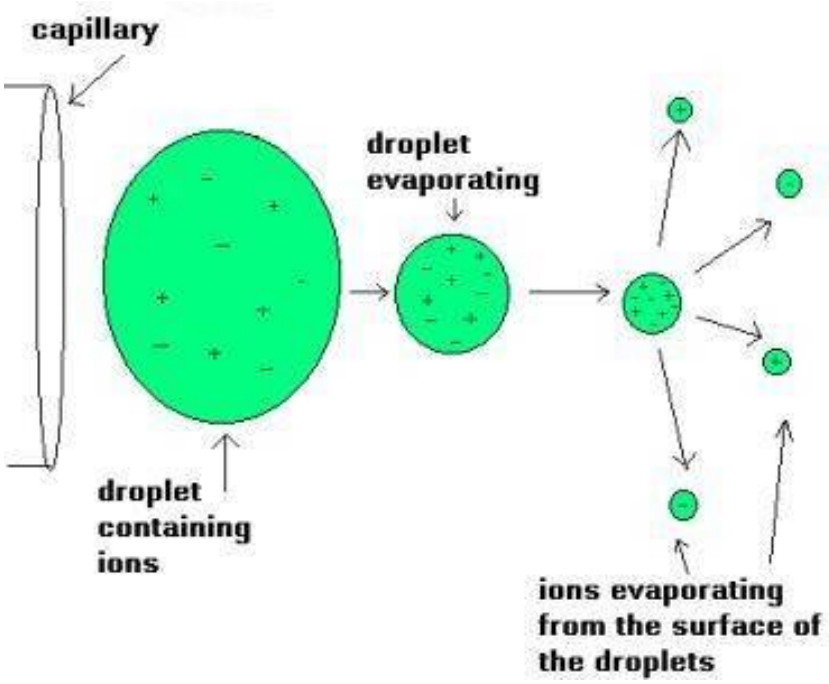
Source: http://upload.wikimedia.org/wikipedia/commons/3/3f/Ionisation_electrospray.png

The smaller the droplets are and thus the less solvent they contain, the more effective desolvation and ionization may be attained. The size of the droplets strongly depends on the flow-rate. When the low flow-rates are applied, the formed droplets are usually smaller and the sensitivity of the analysis higher. The sensitivity of ESI is inversely to a flow-rate of the system [28]. For that reason, low flow-rates are favourable, when using ESI. The flow-rates from 1 μl/min up to several ml/min are typically used [32], most commonly being from 1 μl to 10 μl [29]. Low flow-rates also reduce contamination, as smaller volumes of analyte and buffers are injected [27].

The capillary is usually surrounded by a co-axial flow of nebulizing gas (air or nitrogen) [29]. It is the gas or/and heat that aids the evaporation of the solution from the charged droplets. As the solution evaporates and the droplets become smaller, the repulsion between the charges in the droplet increases [32]. The following process is based on the same principle as the formation of the charged droplets from the Taylor cone. Rayleigh limit is reached, leading this time to an "explosion" of the droplet signed as "coulombic fission". The

process of explosions repeats and as the result, still smaller droplets with fewer charges are created. Finally the ions are completely desorbed to the gas phase. *See Fig.4.*

Fig.4. ESI: Evaporation of the droplet



Source: http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial_files/image006.jpg

The mechanism of the gas phase ions creation is not clear enough. It is considered to be a combination of two mechanisms. The first one to be called Ion evaporation mechanism (IEM) and the second Charged residue model" (CRM). More about these mechanisms can be found in *ref. 34*.

The last step is to bring the gas phase ions to the mass analyzer. It happens due to the appropriate electric field. The transport is provided by an ion transfer capillary, which allows ions from atmospheric pressure to enter the vacuum stage applied in the next parts of the instrument [32]. Moreover a differential pumping system is applied [27]. Here the low flow-rates providing small droplets are again beneficial. Nevertheless, the high flow-rates are sometimes more effective for the chromatographic separation. In that case some auxiliary methods need to be added or geometry of ESI has to be altered, in order to increase sensitivity of the analysis. More can be read in *ref. 26-29 and 32*.

4.3.1.3. ESI and liquid chromatography

Three major incompatibilities meet the ESI-HPLC coupling: i) HPLC usually requires high flow-rates, ii) proportions of the solvent are often changed by the gradient elution, iii) ionization of analyte is suppressed by many buffer additives or pH controllers, such as trifluoroacetic acid (TFA).

The first problem had been more broadly discussed in previous text. The second incompatibility is conveniently resolved by post column mixing of the solution with sheath liquid. The sheath liquid may for example consist of methanol, acetic acid and water in certain concentration ratios [28]. TFA can be replaced with acetic acid. Despite the mentioned difficulties, ESI remains one of the most effective and most often used methods for HPLC coupling.

4.4. Mass Analyzers

Mass analyzers measure the gas phase ions created during the process of ionization. Not the mass itself is measured. The ions are analyzed according to their mass-to-charge ratio.

The principle of the first analyzer designed at the beginning of 20^{th} century was based on the presence of a magnetic field and different curvature of the ions according to their charge. These analyzers have been called magnetic sector analyzers. Even though they might be used nowadays, too, the modern mass analyzers are usually preferred.

The modern mass analyzers are quadrupole (Q), ion trap (IT), time-of-flight and Fourier transform ion cyclotron resonance. They are also very successfully combined in the tandem MS *(see chapter 4.6.)*. All the mass analyzers use static or dynamic electric or magnetic field [27].

Accuracy, resolution, mass range, tandem analysis capabilities, transmissions and scan speed are the frequently used characteristics of mass analyzers performance. These particular characteristics vary from one mass analyzer to another [26].

4.4.1. Quadrupole

Quadrupole (Q) is nowadays the most commonly used mass analyzer. The given characteristics of Q are summarized as follows: accuracy 0.01 %, resolution 4 000, m/z range

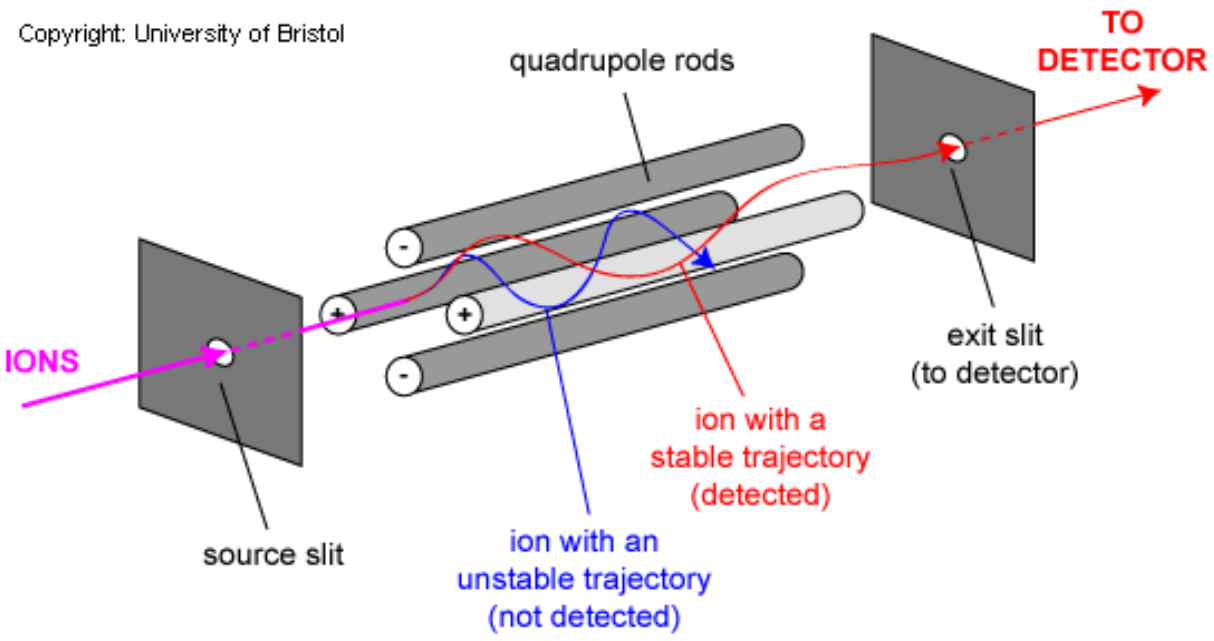
4 000, scan speed within a second, possible MS^2 – the tandem MS itself is characterized with good accuracy as well as resolution and low-energy collisions [26]. Q further offers several advantages. It is tolerable to relatively high pressure and it also has a significant mass range up to 4 000, which is favourable especially for protein analysis.

Q is also called quadrupole mass filter *(see Fig.5.)* The core of this mass analyzer consists of four identical metal rods. The rods are 20 – 30 cm long and have a cylindrical or rather hyperbolic section. They are arranged to the corners of a square. To each pair of opposite standing rods is applied a combination of direct current voltage (DC) and radiofrequency (RF). Meanwhile the value of DC remains the same during the whole analysis, the RF is changed gradually.

The ions are introduced to the space between the pairs of rods and travel through it to the other end, where they meet the detector. There is always only one ion with certain m/z, whose trajectory is stable for the selected RF. This ion passes through the whole space to the detector. All the other ions collide with the rods. RF is changed gradually, until all the ions pass through and are detected. The scanning of the selected m/z range (typically being 100 – 4 000) takes about one second [29].

Q needs a continuous inflow of ions, which might be successfully provided by ESI. Thus their combination becomes very effective [28].

Fig.5. Scheme of quadrupole mass analyzer



Source: http://www.chm.bris.ac.uk/ms/images/quad-schematic2.gif

4.4.2. Ion Trap [35]

They were Steinwedel and Paul, whose work lead to the development of IT mass analyzer. The latter one awarded a shared Nobel Prize for Physics in 1989 [35].

Basically, the IT mass analysers belong to the same group as quadrupole analyzers, regarding their physical principle. Also IT is based on applying RF and DC on electrodes and on motion of ions in defined electric field. Only ions of certain m/z ratio are able to get to the detector. Nevertheless the construction of ion trap is different and slight diversities are found in the motion of the ions within the electric field. More notable differences regard the tandem mass spectrometry principle *(see chapter 4.6.)*.

There are in general two types of ITs: 2D (also called linear IT) and 3D. The first invented IT was the three dimensional one. The analyzer itself consists of three electrodes of hyperbolic shape, one being a ring electrode and the other two being called end-cap electrodes. These are located at the bottom and on the top of the ring electrode.

RF is applied to the ring electrode. As the RF is scanned to higher frequencies, ions caught in the trap oscillate with higher energy. On a certain value of RF, only ion with certain m/z is stable and is send through a small hole in the end-cap electrode to the detector. Herein is the main difference between ion trap and quadrupole visible: meanwhile in Q ions of certain m/z are allowed *to pass* to the detector, in the IT all the ion are present altogether being *expelled* from the trap according to their m/z.

4.5. Detectors

Detailed description of mass spectrometry detectors is behind the scope of this work. Thus only the different types are mentioned: Faraday cup, electron multiplier, channel electron multiplier (CEMA), electro-optical ion detection, the Daly detector and cryogenic detectors [30].

4.6. Tandem mass spectrometry

The main application of tandem MS is more accurate structure determination of compounds. Tandem MS is also referred to as MS/MS or rather MS^n, where *n* indicates

number of transitions. It involves several steps of spectrometry selection, in between which some form of fragmentation occurs.

There are two different approaches to MS^n, first one being tandem-in-space, second tandem-in-time MS. Both of these approaches have their pros and cons and also differ in their applications. In general, tandem-in-space MS is conveyed by several mass analyzers in a row. In between these analyzers is placed a collision cell. The mass analyzers may be either of the same or of different type. Whereas all the steps of tandem-in-time MS take place in one mass analyzer.

4.6.1. Triple quadrupole

The triple quadrupole (QqQ) is a typical example of tandem-in-space MS. Nevertheless, the term itself might be of confusion. The device consists of two quadrupoles, which work as mass analyzers (Q1 and Q3). The middle quadrupole is a collision cell (q2) working in an RF-only mode and it is rather a hexa- or octopole. (To be accurate, even Q1 and Q3 can work in RF-only mode, and thus serve as ion transmission device, as will be explained later in the text.)

The collision cell serves for further fragmentation or dissociation of previously formed ions. The ions react with molecules of gas (most often being a noble gas or nitrogen) forming new ions, which are later analyzed. The process occurring in the collision cell is called collision-induced dissociation (CID) or collision-activated dissociation (CAD). Also other principles are known, but this is the most frequently cited.

4.6.2.1. Scan modes in triple quadrupole

Several scan modes are applicable in QqQ tandem MS: product ion scan, precursor ion scan, neutral loss scan, selected reaction monitoring (SRM.) The Q1 and Q3 either perform scanning in defined m/z range or operate on certain fixed m/z value. More detailed explanation of scan modes can be found in Tab.1.

Tab. 1. Scan modes in tandem MS

Scan mode	*Q1*	*q 2*	*Q3*	*Notes*
Product ion scan	Fixed m/z	CID	Scan	Determination of fragments of certain compound.
Precursor ion scan	Scan	CID	Fixed m/z	Finding compounds giving certain fragment.
Neutral loss scan	Scan	CID	Scan	Ions of m/z equal to the difference of m/z ranges set in Q1 and Q3. Determination of compounds loosing a neutral species (e.g. molecule of water).
SRM	Fixed m/z	CID	Fixed m/z	Only compounds giving the specified fragment are determined.

To make the review complete, there are summarized different data acquisition modes (scan types) used in LC/MS coupling in Tab. 2. For more detailed information about LC/MS *see chapter 4.7.*

Tab. 2. Data acquisition modes in LC/MS

Data Acquisition Mode	*Notes*
Full Scan	The full mass spectrum of a compound is obtained between two extreme masses.
Selected Ion Monitoring (SIM)	Reduces chemical noise, faster, improves detection limit. Several specific ion beams are recorded instead the whole spectrum.
SRM	Even less noise and better detectability than SIM. Can be used only with tandem MS. *See Tab.1.*

4.6.3. Ion Trap tandem MS

The main advantage of IT is the possibility of performing tandem MS in multiple stages. Meanwhile quadrupole was limited with MS^2 (two stages of MS), in ion trap MS^n can be performed. This undoubted advantage is nevertheless counterbalanced by several limitations.

The first one is the disability to perform precursor ion and neutral loss scan. „One third rule“ represents another limitation. According to this rule the upper limit of the ratio between m/z of precursor ion and m/z of the lowest trapped ion is 0.3. (E.g. if the m/z of precursor ion is 1 200, the fragment ions will not be detected under m/z 400.) The third limitation lies in the fact, that the dynamic range of IT can be limited when too many ions are present in the trap. This can be avoided by counting the ions entering the trap and prevent entrance of further ions once the limit is reached.

The principle of tandem MS process in IT is though very simple: ion of certain m/z ratio is selected by expelling all the other ions from the trap, where all the ions are present together. The remained ion undergoes a fragmentation (based on a collision with gas – CID) and these fragments pass gradually to the detector according to the applied RF. When multiple stages tandem MS is performed, from all the obtained fragments again only one certain is held in the trap, the others being expelled. So the process can continue with desirable stages of tandem MS. However with increasing number of MS^n stages the sensitivity gets lower and more concentrated samples are needed.

4.7. LC/MS – Liquid Chromatography coupled with Mass Spectrometry [36]

4.7.1. HPLC

The acronym HPLC stands for High-Performance Liquid Chromatography. It is a known separation technique often used in analytical labs. It has evolved from GC at the beginning of 1970’. During the four decades, the development of HPLC has made a huge progress. Nowadays the developing efforts are aimed particularly in miniaturizing of the technique.

The main advantages in comparison to GC comprise a broad application field. HPLC is suitable for analyses of ions, polar and non-polar compounds, non-volatile as well as high-molecular compounds. The separation itself can be influenced by the composition of mobile phase. On the other hand some of the disadvantages are represented by more complicated instrumentation and mechanism of the separation.

4.7.2. Interfaces

There are several difficulties meeting the coupling of LC with MS. The fundamental considerations are i) an efficient removal of mobile phase, ii) relatively high flow rates used in LC, iii) LC is used for analyses of non-volatile or polar compounds, which cannot be analyzed by GC as their transfer to gas phase (a crucial step in MS) is either difficult or even impossible.

The cited obstructions were resolved in the past by several mechanisms. They were: moving belt interface, direct liquid introduction, thermospray, Fast Atom Bombardment (FAB) and particle beam interface [36]. In the present the most often used interface for successful coupling of LC with MS is the atmospheric pressure ionization, with ESI as the main representative [30].

Another limitation for LC/MS coupling comprises the composition of a mobile phase. Several practical recommendations should be thus followed: i) minimizing content of non-volatile constituents in mobile phase, ii) avoiding the use of Na^+ and K^+ salts, as they may cause quick clogging of ionization source, iii) preferring methanol to acetonitrile.

5. Background of the current project

5.1. Key reference: Amino acids model assay

The key reference and the fundamental background of this thesis is *ref. 1: Mechanistic Proposal for the Formation of Specific Immunogenic Complexes via a Radical Pathway: A Key Step in Allergic Contact Dermatitis to Olefinic Hydroperoxides. Staffan Johansson, Theres Redeby, Timothy M. Altamore, Ulrika Nilsson and Anna Börje. Chem. Res. Toxicol. 2009, 22, 1774–1781*.

The paper concerns the reactivity of limonene-2-hydroperoxide (Lim-2-OOH). It investigates the mechanism of formation of specific immunogenic complexes of Lim-2-OOH. The research was realized by studying the formation of adducts between Lim-2-OOH and protected amino acids/glutathione in the presence of Fe (III) porphyrin complex. The mechanism of formation was examined and structures of the isolated adducts were determined or at least suggested.

The research comprised: i) synthesis of protected amino acids, Lim-2-OOH, carvone and carveol (two compounds formed from Lim-2-OOH in the presence of Fe (III) porphyrin complex), ii) performing the reactions between Lim-2-OOH, carvone and carveol and the protected amino acids/glutathione in the presence/absence of Fe (III) porphyrin complex, iii) fractionation of the reaction mixtures by preparative HPLC, iv) detection of possible adducts formation in the fractionated reaction mixtures (screening experiments) using LC/MS, v) structural analysis of the isolated adducts by LC/MS and two dimensional NMR experiments.

5.2. Further research: Peptides model assay

Forming of the hapten-protein adducts of Lim-2-OOH was also examined in peptides-model assay (*ref. 37*). Human angiotensin I and bovine insuline were used in this experiment. The results showed that the haptenation of the proteins is eventually enabled just in the presence of Fe (III) porphyrin complex as a radical initiator. In the absence of the complex, no reaction took place. This finding supports the hypothesis that a radical mechanism is involved.

Angiotensin I showed a monohaptenation in the reaction with Lim-2-OOH. Further more detailed examination of the haptenation process showed, that the angiotensin was haptenated through histidine molecule in its amino acids chain. Also insulin showed adducts formation in reaction with Lim-2-OOH. Histidine was again the haptenated amino acid. Moreover, possibility of binding to the cysteine (generated from the redox conditions of the model system) was also suggested.

5.3. Limonene-1-hydroperoxide x Limonene-2-hydroperoxide

Limonene as a monoterpene with double bonds is prone to self-oxidation. After air exposure it forms a mixture of oxidation products. Among them the limonene hydroperoxides were proven to be strong skin sensitizers.

5.3.1. Allergic potential

Sensitizing capacity of both naturally formed hydroperoxides (Fig.2. in chapter 2.3.) was examined in local lymph node assay (LLNA). This animal assay is based on incorporating radioactive thimidine by local lymph nodes, which are proliferating, when the immune system of the animal react to injected chemical. The more thimidine is detected, the more the immune system is activated and thus the higher sensitizing capacity the chemical has. The animals used during this test are mice and the tested chemical is applied on the back of their ears. The final outcome is expressed as stimulation index. When the stimulation index is higher than 3, the chemical is considered to give positive result in LLNA. The values of stimulation index are then calculated to get EC3 values. The lower EC3 value is the stronger sensitizer the tested chemical is. There are four categories: extreme, strong, weak and non-sensitizers [2, 3].

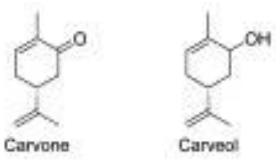
EC3 values of the examined hydroperoxides were as follows: for limonene-1-hydroperoxide (Lim-1-OOH) 0.33 % and for Lim-2-OOH 0.83 %. A chemical is classified as a strong skin sensitizer, if the EC3 value is between 0.1 and 1.0 %. That makes both limonene hydroperoxides chemicals with strong sensitizing capacity. And when the two of them are compared, Lim-1-OOH shows to be stronger skin sensitizer [3].

5.3.2. Structure

The structure of limonene hydroperoxides is depicted on Fig.2. in chapter 2.3. From the formulas is obvious, that Lim-1-OOH is a tertiary hydroperoxide, meanwhile Lim-2-OOH is a secondary hydroperoxide. Noticeably due to their slightly different structure they will create both: different radicals and thus different adducts with amino acids, proteins eventually.

The distinction in the structure has another aspect: it was shown, that Lim-2-OOH forms in the presence of Fe (III) porphyrin complex other compounds. The mostly formed degradation product was carvone together with a small amount of carveol. Both of them are likely to form adducts with amino acids [1].

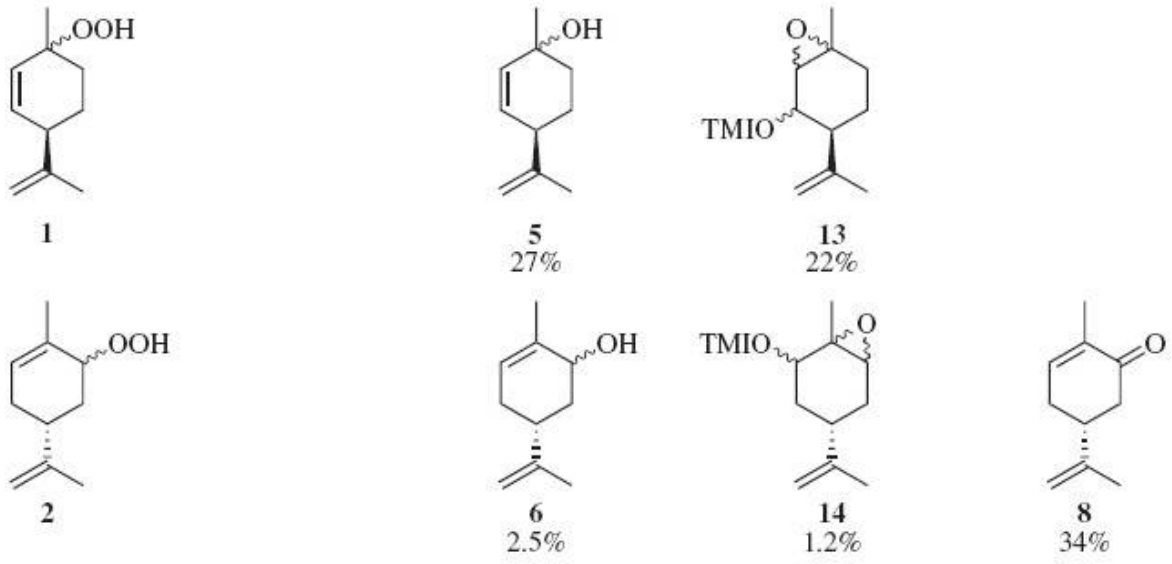
Fig.6. Carvone and carveol



5.3.2.1. Radical formation

In chapter 3.2.1 the radical formation of limonene-hydroperoxides was discussed. Oxygen-centered allyloxyl radicals formed by homolytical cleavage of O-O bond. Further rearrangement and reactions of these allyloxyl radicals produced new radicals together with nonradical products. There are three different pathways of O-centered radicals rearrangement into carbon centered radical: i) hydrogen abstraction (resulting in analogous alcohol), ii) 1,2-shift (generating carbon-centered R-hydroxyl radical) and iii) 1,3-cyclization (yielding the creation of primary or secondary carbon-centered alkyl radicals) [24]. Considering the differences in limonene hydroperoxides structure, also different radicals are formed. Production of different radicals will influence formation of immunogenic complexes between the hydroperoxide and skin proteins in ACD process. These radicals should be available for the formation of immunogenic complexes [24].

Fig.7. Comparison of radical formation betweenLim-1-OOH (1) and Lim-2-OOH (2) [3].



When structure of the radicals was examined, radical trapping experiments with 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO) as a radical scavenger, creating stable

nonradical products were applied. In the radical trapping experiment with Lim-1-OOH two major products were isolated and their structure has been revealed by NMR experiments. They were the corresponding alcohol (5) and TMIO adducts of an oxiranylcarbinyl radical (13). In the radical trapping experiment with Lim-2-OOH, the products isolated and characterized were carvone (8), carveol (6) and the TMIO adduct of the oxiranylcarbinyl radical (14).

When considering the three pathways of radical formation (*see chapter 3.3.*), the pathway ii) in Lim-1-OOH reaction is blocked by the methyl group on the carbon bearing the hydroperoxide functionality. Instead, the allyloxyl radical is enabled to follow pathways i) and iii). On the contrary the allyloxyl radical formed in the experiment with Lim-2-OOH was shown to be able to follow all three pathways. However, pathway ii) is likely to dominate in the radical formation procedure [3, 24].

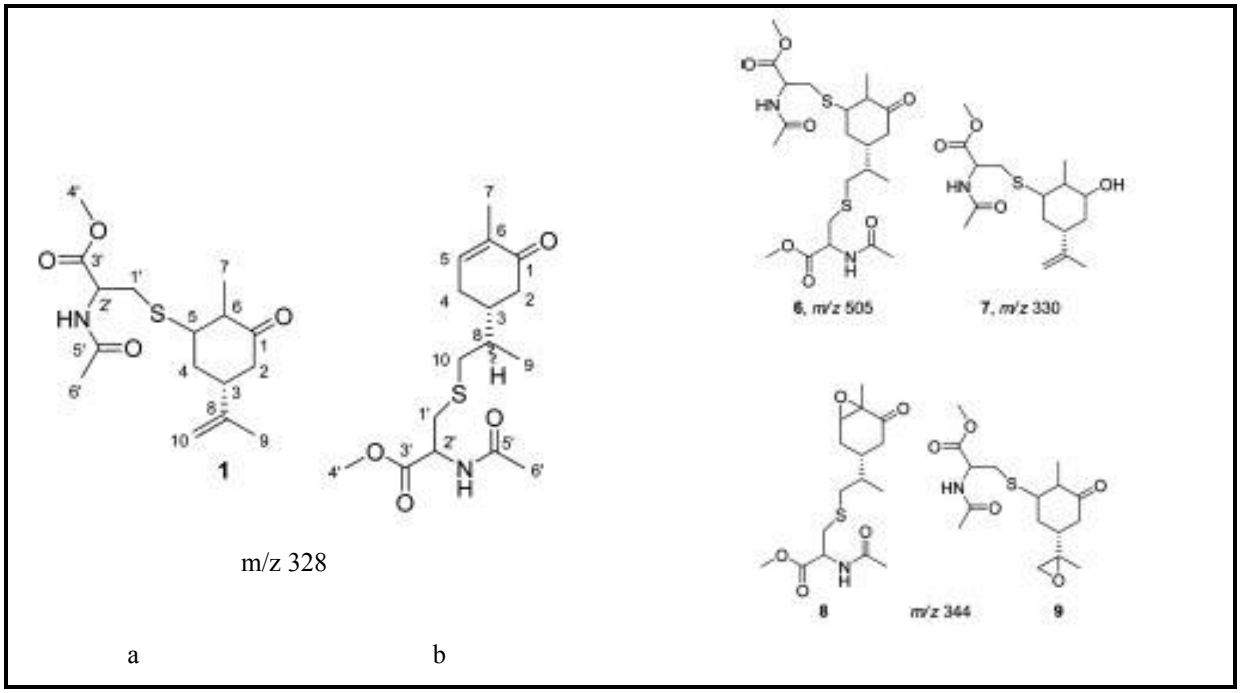
5.3.2.2. Amino acids adducts formation

In the *ref. 1 and 37*, formation of adducts between Lim-2-OOH and amino acids or peptides model respectively, was investigated. The results showed that Lim-2-OOH creates adducts with cysteine. Histidine formed small amounts of likely adducts, meanwhile no adducts were observed with alanine, leucine, tryptophane, tyrosine nor lysine [1]. In *ref. 37*, possible adduct formation between Lim-2-OOH and proteins through binding to histidine was confirmed.

The adducts were detected only when the reaction were run in the presence of Fe (III) porphyrin complex, strongly supporting the radical mechanism of adducts formation. Structure of isolated adducts were scrutinized using LC/MS and NMR and the final results are summarized Fig.8. [1].

In the reactions of Lim-2-OOH with cysteine in the presence of Fe (III) porphyrin complex, firstly the oxygen-oxygen bond is cleaved homolyticaly forming allyloxyl radical. This radical is transformed in further reaction into carbon-centered radicals as well as to carvone and carveol. Cysteine contains in the structure thiol functional group. Thiyl radical can be formed, when any of the radicals formed from Lim-2-OOH abstract hydrogen. Thiyl radicals may then react with olefinic double bonds in thiol-ene reaction, forming the adducts 1a, 1b, 6 and 7. Adducts 8 and 9 correspond to adducts with one extra oxygen.

Fig.8. Adducts formed between Lim-2-OOH and protected cysteine [1]



PART 2 - Experimental

6. Aim of the thesis

Allergic contact dermatitis is a serious problem of the modern society. It is a chronic disease. ACD is a hypersensitivity reaction which makes the immune system being involved. It manifests itself as a skin rash or eczema after a sensitized person is exposed to certain chemical species.

Limonene is a fragrance compound used in perfumes and household products. As a monoterpene with double bonds, it is prone to self-oxidation after air exposure. A mixture of limonene oxidation products is created during the process. Among the oxidation products limonene hydroperoxides were found to be strong skin sensitizers.

The allergenic compounds (haptens) causing ACD react with skin proteins. The bond between the hapten and the protein is usually covalent and in most cases is based on electrophilic-nucleophilic interactions. The hydroperoxides are nonetheless believed to haptenate the skin proteins in radical mechanism.

The formation of radicals originating from limonene hydroperoxides was examined. The pathways of the radical's creation and their structure have been identified. For better understanding the ACD process is crucial to know more about the mechanism of haptenation i.e. about the interactions between the haptens and the skin proteins.

In recently the reactivity of Lim-2-OOH with amino acids (*ref. 1*) and with model peptides (*ref. 37*) was examined. Adducts between Lim-2-OOH and amino acids or peptides respectively were formed in the presence of Fe (III) porphyrin complex used as a radical initiator. Adducts with amino acids were isolated and their structure was revealed or at least suggested using LC/MS.

Lim-1-OOH was found to be stronger skin sensitizer from the two limonene hydroperoxides. In this thesis the reactivity of Lim-1-OOH is therefore aimed. The intention was to reveal, whether Lim-1-OOH react in the amino acid models assay in the same fashion as Lim-2-OOH and suggest the structures of the estimated adducts.

The project involved:

i) Screening experiments - analyses of complex reaction mixtures between Lim-1-OOH and amino acids in the presence of Fe (III) porphyrin complex. This part of the research revealed, which amino acids potentially create adducts with

Lim-1-OOH. Nevertheless they were just rudimentary results. For expelling the falsely positive results, the next stage had to be performed.

ii) Control samples experiments – the control samples were reaction mixtures of Lim-1-OOH and amino acids without the Fe (III) porphyrin complex. They were prepared and analyzed by LC/MS. The structure of the adducts formed only in the presence of the radical initiator was about to be examined. Therefore structures of certain m/z, which were seen in both samples (control and original) were not considered to be relevant adducts, that is those formed via a radical pathway.

iii) MS^n experiments – after elimination of the non-adduct m/z MS^n experiments were focused only on created adducts. In the last stage of the project the structure of the Lim-1-OOH adducts were revealed or at least suggested.

7. Instrumentation

The original intention was to use one of the most sensitive instruments which the Department of analytical chemistry at Stockholm University could offer: TSQ Vantage (Thermo Scientific) equipped with a triple quadrupole. For tuning the instrument and for optimizing the methods, the samples from Lim-2-OOH experiments were used. They were namely: adduct of carvone and cysteine (m/z 328) and protected cysteine (m/z 178). The separation was demonstrated by mixing the two samples together and afterwards running the mixture through a reverse phase-based column.

Unfortunately the work was slowed down by a disability to separate both compounds - cysteine and adduct. Moreover, when either isocratic elution with 5 % of acetonitrile (ACN) in water or 5 % of water in ACN was applied, the same retention times (approx. two minutes) were observed. After trying several different columns, the suggestion was that these problems might have been caused by the initial dissolving of the samples in pure ACN. This might have brought along the situation, when the compounds would not stuck on the reverse phase-based column. Therefore the samples were resuspended in water. However, not even after the resuspension clear separation was gained.

At last, the instrument was changed. LCQ Finnigan DECA equipped with ion trap was involved. The desired separation on LCQ DECA was reached without any problem and after

setting the most favourable conditions, the samples of Lim-1-OOH reaction mixture could have been applied.

Later during the research TSQ Vantage instrument was involved again. Nevertheless, the separation problems persisted on TSQ Vantage, when the autosampler was implied. Clear separation was observed only during manual injection of the samples. However, in the case of manual injection, gradient elution could not be exploited. Therefore during the experiments on TSQ Vantage, isocratic elution was performed. This fact did not cause any troubles regarding the credibility of the results.

7.1. LCQ Finnigan DECA

- Mass analyzer: ion trap
- Settings: sheath gas flow rate 40 arb, auxiliary gas flow rate 15 arb, spray voltage 5kV, capillary voltage 35 V, tube lens offset -35 V, capillary temperature 250 °C
- Column: X-Terra MS C18, 2.5um, 3.0 x 50 mm
- Mobile phase: solvent A (95 % water, 5 % acetonitrile, 0.1 % of formic acid), solvent B (95 % acetonitrile, 5% water, 0.1 % formic acid)
- Gradient: 20 minutes of 100 % to 0 % A in B
- Flow rate: 100 µl/min

7.2. TSQ Vantage

- Mass analyzer: triple quadrupole
- Settings: spray voltage 3000 V, vaporizer temperature 200 °C, sheath gas pressure 30 arb, ion sweep gas pressure 0.0 arb, aux gas pressure 10 arb, capillary temperature 250 °C, S-Lens RF amplitude 110, declustering voltage -0 V
- Column: X-Terra MS C18, 2.5um, 3.0 x 50 mm
- Mobile phase: solvent A (95 % water, 5 % acetonitrile, 0.1 % of formic acid)
- Isocratic elution with 100% A
- Flow rate: 200 µl/min

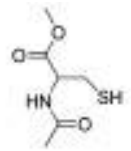
8. Screening experiments

The screening experiments included the LC/MS analyses of reaction mixtures of Lim-1-OOH and protected amino acids in the presence of Fe (III) porphyrin complex.

8.1. Amino acids

The amino acids were chosen to comprise these with typically nucleophilic properties. There were seven used amino acids: alanine (ala), leucine (leu), tryptophane (trp), tyrosine (tyr), cysteine (cys), histidine (his) and lysine (lys). All the amino acids were protected on both amino and carboxyl group to mimic their properties in a protein chain. The amino group was substituted with acetyl group ($COOCH_3$), the carboxyl group was esterified with methanol. Histidine was the only exception, as the amino group was in this case protected with benzoyl.

Fig.9 NAc-cysteine-OMe



8.2. Reaction mixtures

The reaction mixtures were obtained from the Department of Chemistry, Dermatochemistry and Skin Allergy, University of Gothenburg, Gothenburg, Sweden. On Fig.10 is depicted the whole process of sample preparation. For LC/MS analyses was used only the water phase.

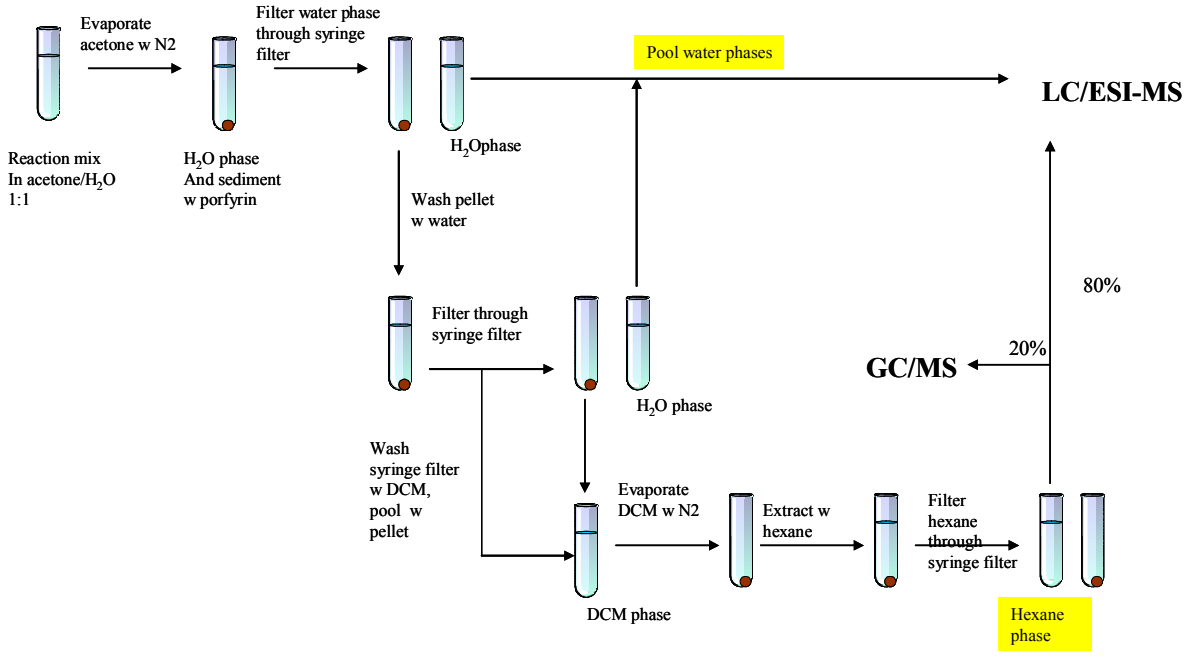
All the samples were diluted 10 and 100 times using solvent A (10 µm of mixture + 90 µm of solvent A, i.e. 95 % water, 5% ACN and 0.1% of HCOOH). The reaction mixtures of tryptophane, tyrosine and histidine contained small particles. These samples were therefore filtrated using a TITAN polypropylene 0.45µm filter. The filter and the syringe with sticked particles were stored in a freezer for possible later use.

After running every single reaction mixture, several protonated masses of the potential predicted adducts were focused. The masses were chosen according to the following pattern:

sum of the mass of the particular protected amino acid and mass of Lim-1-OOH plus proton (amino acid + Lim-1-OOH + H^+). The other alternatives are then plus/minus one or two oxygens. Each of the possible adducts also in without-two-hydrogens option. From the University of Gothenburg also came suggestions of masses of possibly formed adducts including the structure.

Another strong reason for aiming the particular m/z was its intensity in the total ion current (TIC) and/or relatively high intensity in extracted ion current (RIC). Formation of narrow sharp peak was also considered to be a sign for closer observation of the m/z.

Fig.10. Sample preparation



Flow schedule for sample prep that has been performed of crude h per –aminoacid reaction mixtures. The step in green still is to be done.

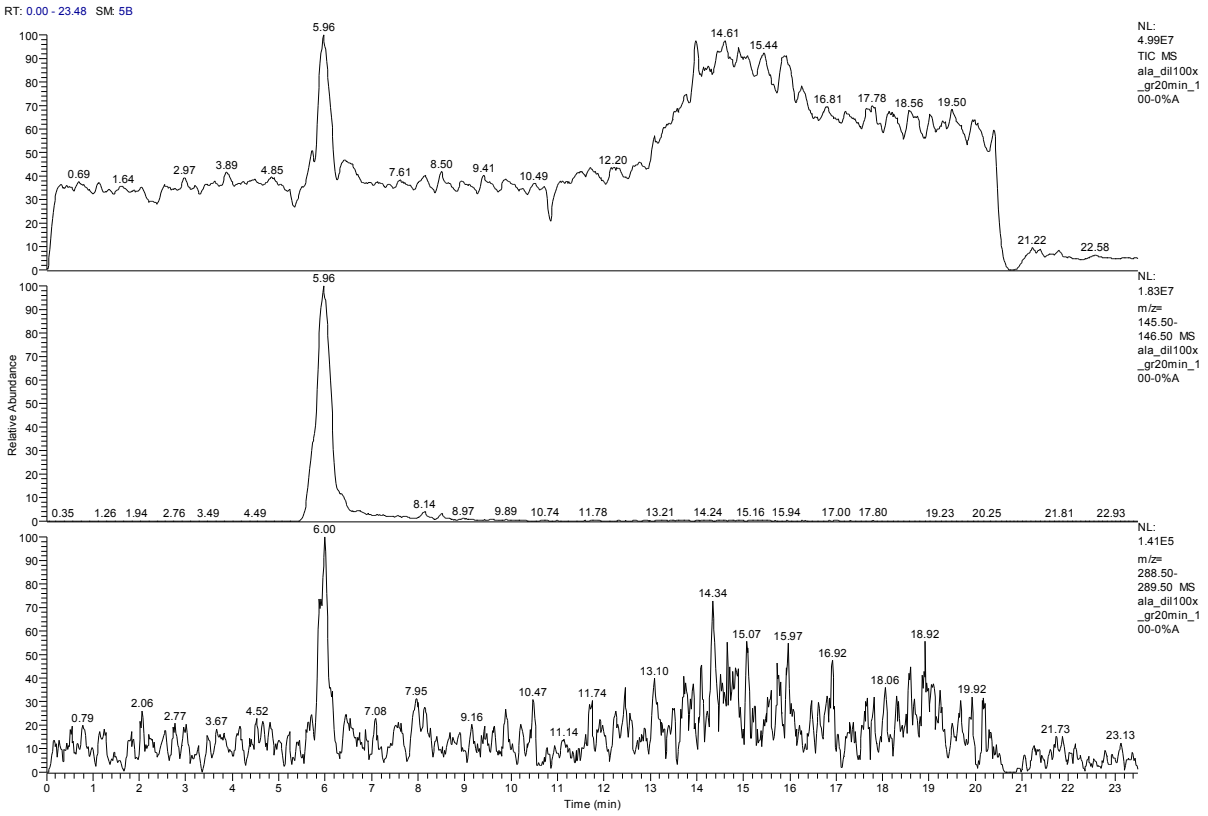
8.3. Alanine

Results of the 100 times diluted sample are shown. The screening experiments were performed on LCQ DECA. Intensity TIC was 4.99.E7 counts. The chromatogram of alanine reaction mixture showed one intense narrow peak at the retention time (RT) 5.96 min, which stands for the protected alanine itself (m/z 146). The other protonated masses, which were predicted as possible adducts, were not detected at high intensities, nor were narrow peaks seen. The protonated mass 167 showed on the contrary nice narrow peak of relatively high intensity. Intensities of the estimated adducts are summarized in Tab.3.

Tab.3. Intensities of RICs in alanine reaction mixture diluted 100x

Protonated mass	*Intensity in RIC*	*Note*
146	**1.83 E7**	**protected ala**
289	**1.41 E5**	**dimer of ala**
314 Ala + Lim-1-OOH + H^+	7.47 E4	
312 Ala + Lim-1-OOH – 2H + H^+	8.84 E4	
330 Ala + Lim-1-OOH plus O + H^+	7.51 E4	
328 Ala + Lim-1-OOH plus O – 2H + H^+	8.90 E4	
346 Ala + Lim-1-OOH plus 2 O + H^+	9.39 E4	
344 Ala + Lim-1-OOH plus 2 O – 2H + H^+	1.33 E5	
298 Ala + Lim-1-OOH minus O + H^+	9.96 E4	
296 Ala + Lim-1-OOH minus O – 2H + H^+	1.47 E5	
282 Ala + Lim-1-OOH minus 2 O + H^+	8.50 E4	
280 Ala + Lim-1-OOH minus 2 O – 2H + H^+	9.86 E4	
167	**1.33 E6**	narrow peak of relatively high intensity

On the top: TIC of alanine reaction mixture diluted 100x, in the middle: extracted ion current (RIC) of m/z 146 (ala), at the bottom: RIC of m/z 289 (dimer)

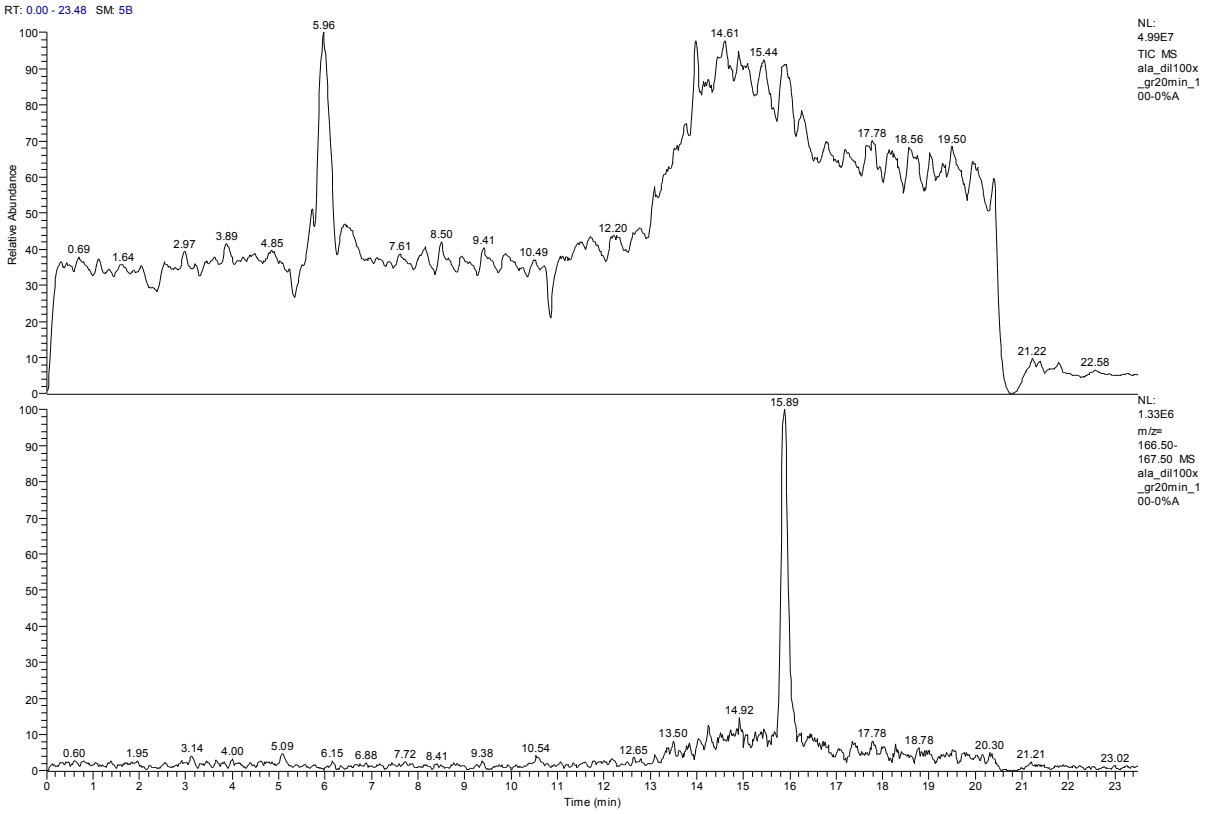


Conclusions

1. Amount of the created dimer (m/z 289) is under average considering the other amino acids.
2. From the University of Gothenburg came no suggestions about the possible adducts formation.

3. The 167 protonated mass could be in correlation with Lim-1-OOH (m/z 169) but is definitely not adduct of the hydroperoxide and protected alanine. It could be a degradation product of the hydroperoxide when subjected to a radical initiator.

On the top: TIC of alanine reaction mixture diluted 100x, at the bottom: RIC of m/z 167



→ **Lim-1-OOH is not considered to create any likely adducts with alanine.**

8.4. Leucine

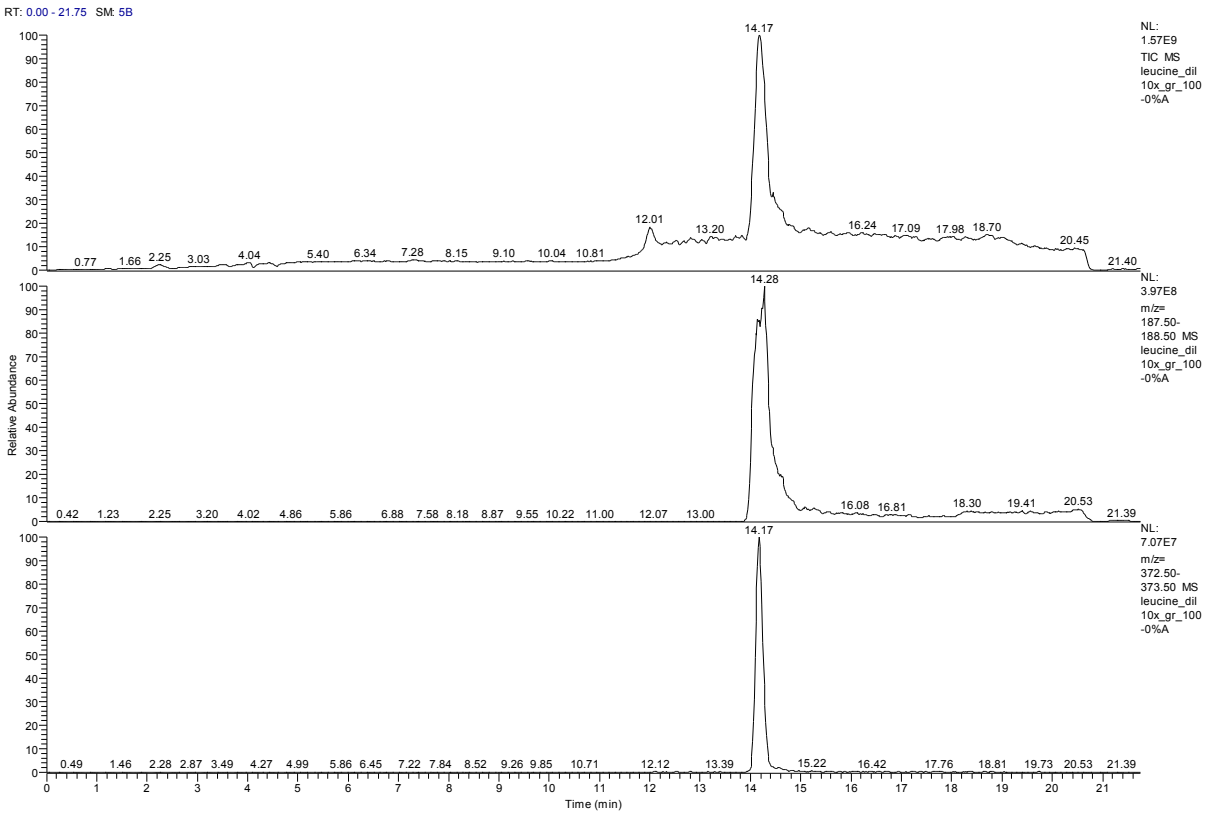
Results of the 10 times diluted sample are shown. The screening experiments were performed on LCQ DECA. Intensity of TIC was 1.57 E9 counts. Leucine showed narrow intensive peak in RT 14.28 min, which corresponds to the protected leucine (m/z 188) itself. M/z of leucine dimer (m/z 373) was also of high intensity. The protonated masses 354 and 372 were suspected to be adducts, nevertheless the intensity of RIC was too low to make these two being of relevant interest. The 353 protonated mass was on the contrary about to be examined more deeply. Resume of particular RIC intensities are shown in Tab. 4.

Tab.4. Intensities of RICs in leucine reaction mixture diluted 10x

Protonated mass	*Intensity in RIC*	*Note*
188	**3.97 E8**	**protected leu**
373	**7.07 E7**	**dimer of leu**
356 Leu + Lim-1-OOH + H^+	1.46 E6	
354 Leu + Lim-1-OOH – 2H + H^+	**5.67 E6**	✓
372 Leu + Lim-1-OOH plus O + H^+	**2.83 E6**	✓
370 Leu + Lim-1-OOH plus O – 2H + H^+	1.42 E6	
388 Leu + Lim-1-OOH plus 2 O + H^+	2.26 E6	
386 Leu + Lim-1-OOH plus 2 O – 2H + H^+	8.95 E5	
340 Leu + Lim-1-OOH minus O + H^+	2.58 E6	
338 Leu + Lim-1-OOH minus O – 2H + H^+	1.72 E6	
324 Leu + Lim-1-OOH minus 2 O + H^+	1.05 E6	
322 Leu + Lim-1-OOH minus 2 O – 2H + H^+	9.41 E5	
353	**3.36 E7**	✓ higher intensity

N.B. ✓ stands for relatively straight narrow peak, **possible adduct**

On top: TIC of leucine reaction mixture diluted 10x, in the middle: RIC of m/z 188 (leu), at the bottom: RIC of m/z 373 (dimer)

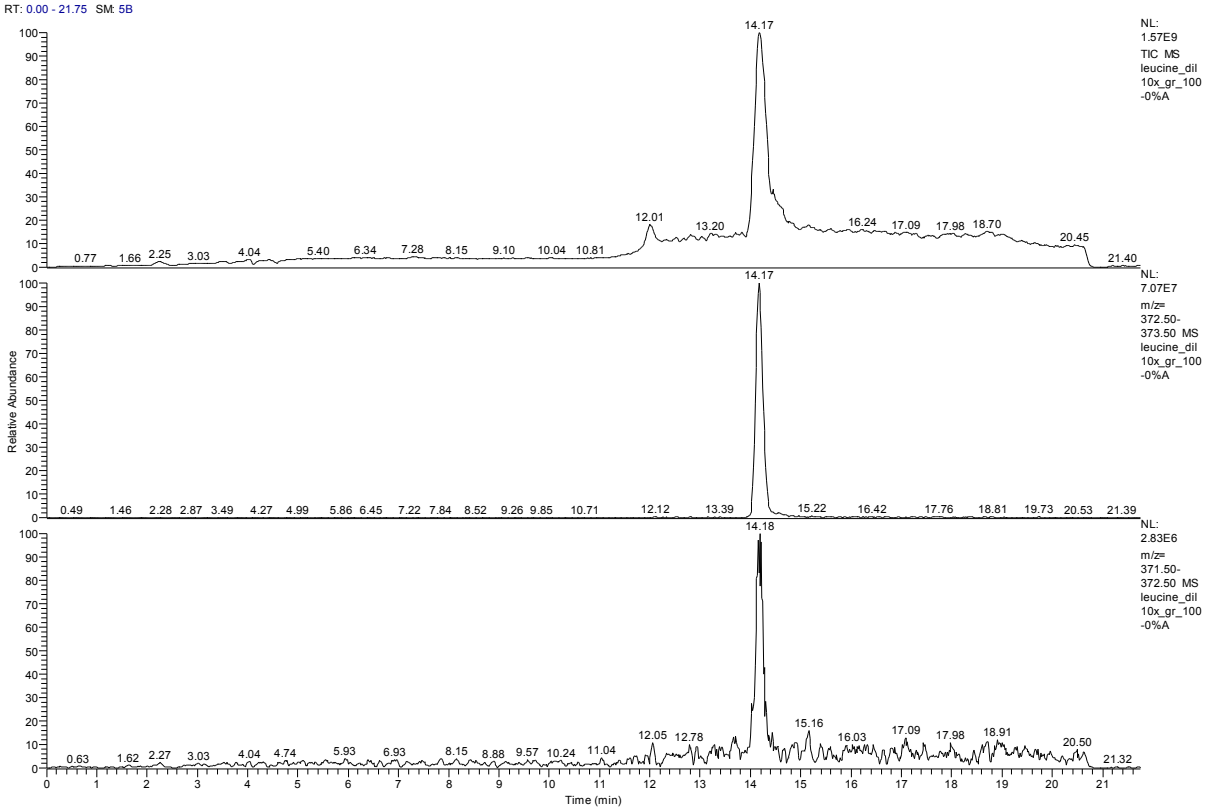


Conclusions

1. Amount of the created dimer (m/z 373) is average considering the other amino acids.
2. From the University of Gothenburg came no suggestions about the possible adducts formation.

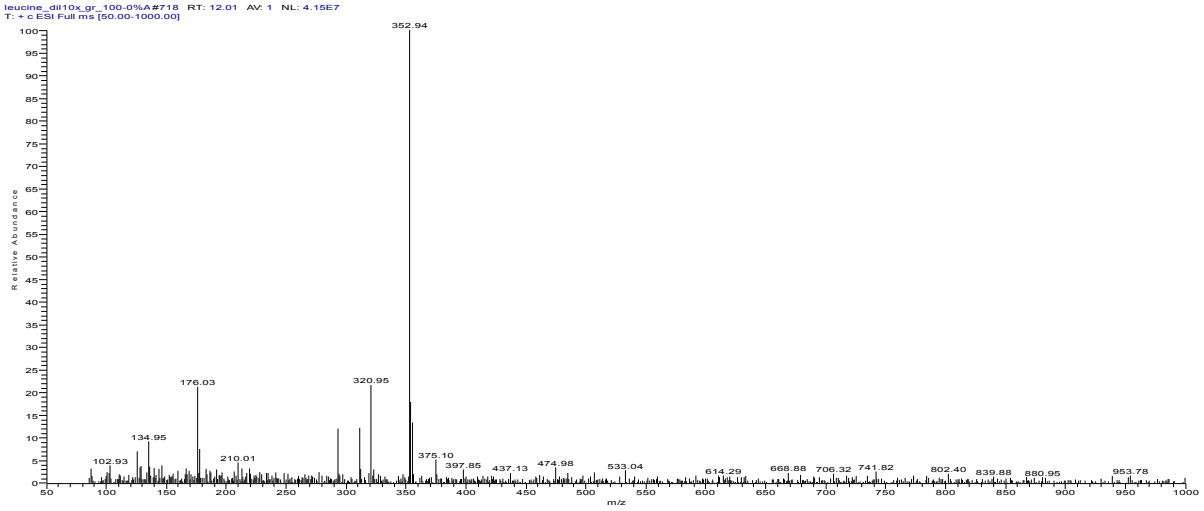
3. The protonated mass 372 had the same occurrence as the leucine dimer (m/z 373). The intensity of the dimer was higher (*see Tab.4.*). The 372 protonated mass is therefore not considered to be adduct of Lim-1-OOH.

On the top: TIC of the leucine reaction mixture diluted 10x, in the middle: RIC of m/z 373 (dimer), at the bottom: RIC of m/z 372



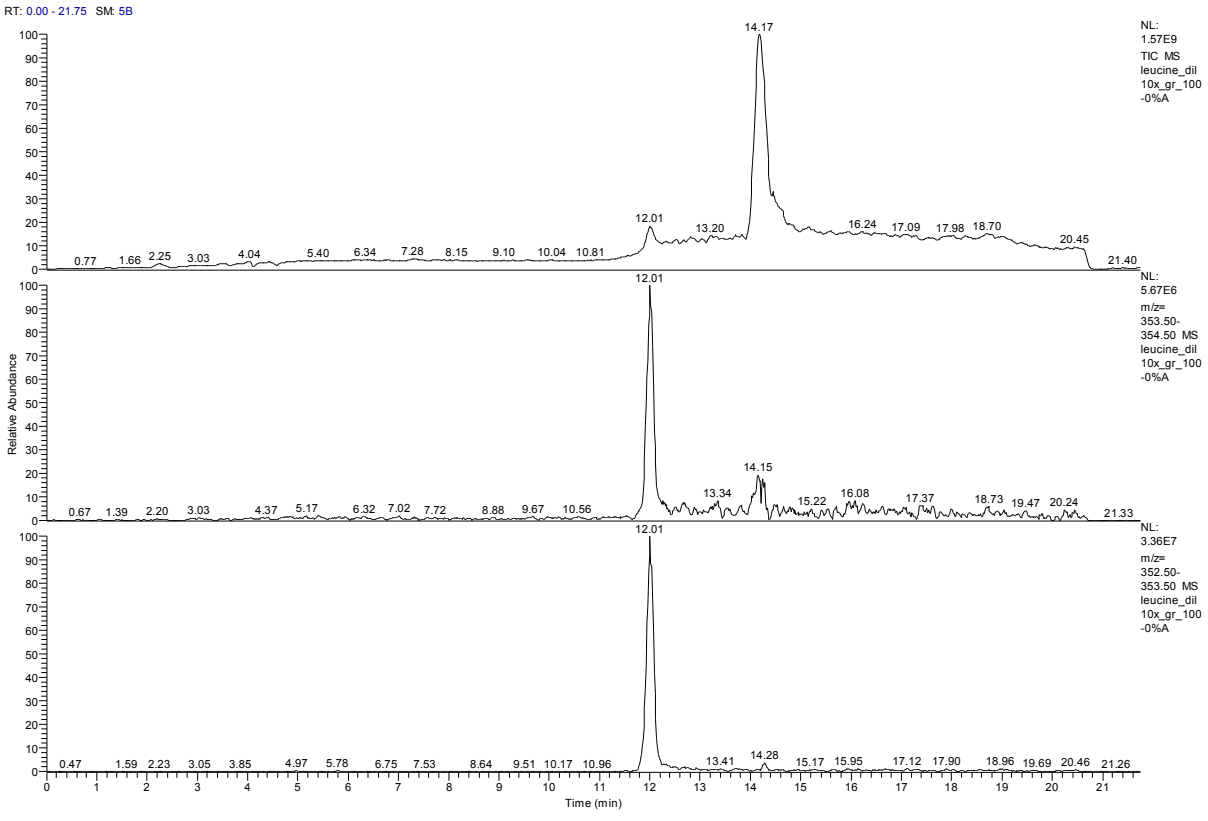
4. The protonated mass 353 showed small peak in RT 12.01 min.

RT 12.01



The peaks of the protonated masses 353 and 354 also had the same position in the chromatogram (RT 12.01 min). From the two of them 353 was of higher intensity (*see Tab.4.*).

On the top: TIC of the leucine reaction mixture diluted 10x, in the middle: RIC of m/z 353, at the bottom: RIC of m/z 354



When MS^n experiments of the 353 protonated mass of leucine were performed, the same fragments as of the cysteine dimer (m/z 353) were observed.

MS^2 of cysteine dimer (353), sample diluted 10x:
176.00, 209.84, 292.90, 320.87, 335.29

MS^2 of the 353 protonated mass seen in leucine sample diluted 100x:
176.09, 209.85, 279.00, 292.80, 320.84, 336.26

There is no satisfying explanation of how the cysteine dimer could have gotten to the leucine reaction mixture, however the 353 protonated mass is not considered to be any relevant adduct of Leucine with Lim-1-OOH. Because of the narrow correlation between 353 and 354 protonated masses, neither 354 is considered to be the formed adduct.

→ **Lim-1-OOH is not considered to create any likely adducts with leucine.**

8.5. Tryptophane

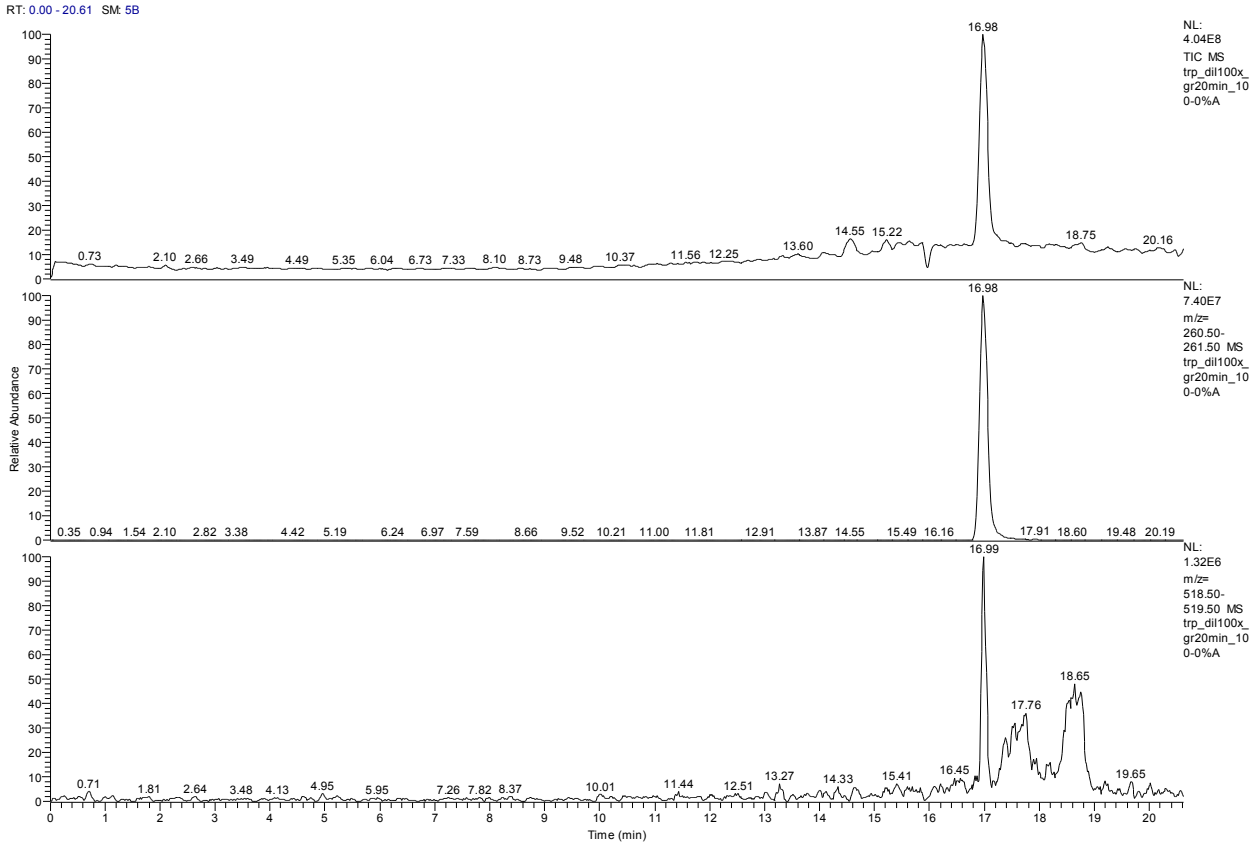
The results of the 100 times diluted reaction mixture of tryptophane are summarized in following text. The screening experiments were performed on LCQ DECA. The intensity of TIC was 4.04 E8 counts. One intensive peak was observed at RT 16.98 min. This peak belongs to the protected tryptophane (m/z 261) itself. The dimer (m/z 519) of tryptophane was less intense and had the same position in the chromatogram. The results are summarized in Tab.5.

Tab.5. Intensities of RICs in tryptophane reaction mixture diluted 100x

Protonated mass	*Intensity in RIC*	*Note*
261	**7.40 E7**	**protonated trp**
519	**1.32 E6**	**dimer of trp**
429 Trp + Lim-1-OOH + H^+	3.55 E5	
427 Trp + Lim-1-OOH – 2H + H^+	2.14 E5	
413 Trp + Lim-1-OOH minus O + H^+	8.66 E5	estimated adduct from the University of Gothenburg
411 Trp + Lim-1-OOH minus O – 2H + H^+	4.81 E5	estimated adduct from the University of Gothenburg
369	2.75 E5	estimated adduct from the University of Gothenburg
397 Trp + Lim-1-OOH minus 2 O + H^+	**8.65 E5**	✓ low intensity
395 Trp + Lim-1-OOH minus 2 O – 2H + H^+	7.39 E5	
445 Trp + Lim-1-OOH plus O + H^+	2.97 E5	
443 Trp + Lim-1-OOH plus O – 2H + H^+	**1.57 E6**	✓ low intensity
461 Trp + Lim-1-OOH plus 2 O + H^+	2.05 E5	
459 Trp + Lim-1-OOH plus 2 O – 2H + H^+	**7.67 E5**	✓ low intensity
277	**3.59 E6**	relatively intensive narrow peak.

N.B. ✓ stands for relatively straight narrow peak, **possible adduct**

On the top: TIC of the tryptophane reaction mixture diluted 100x, in the middle: RIC of m/z 261 (trp), at the bottom: RIC of m/z 519 (dimer of trp).

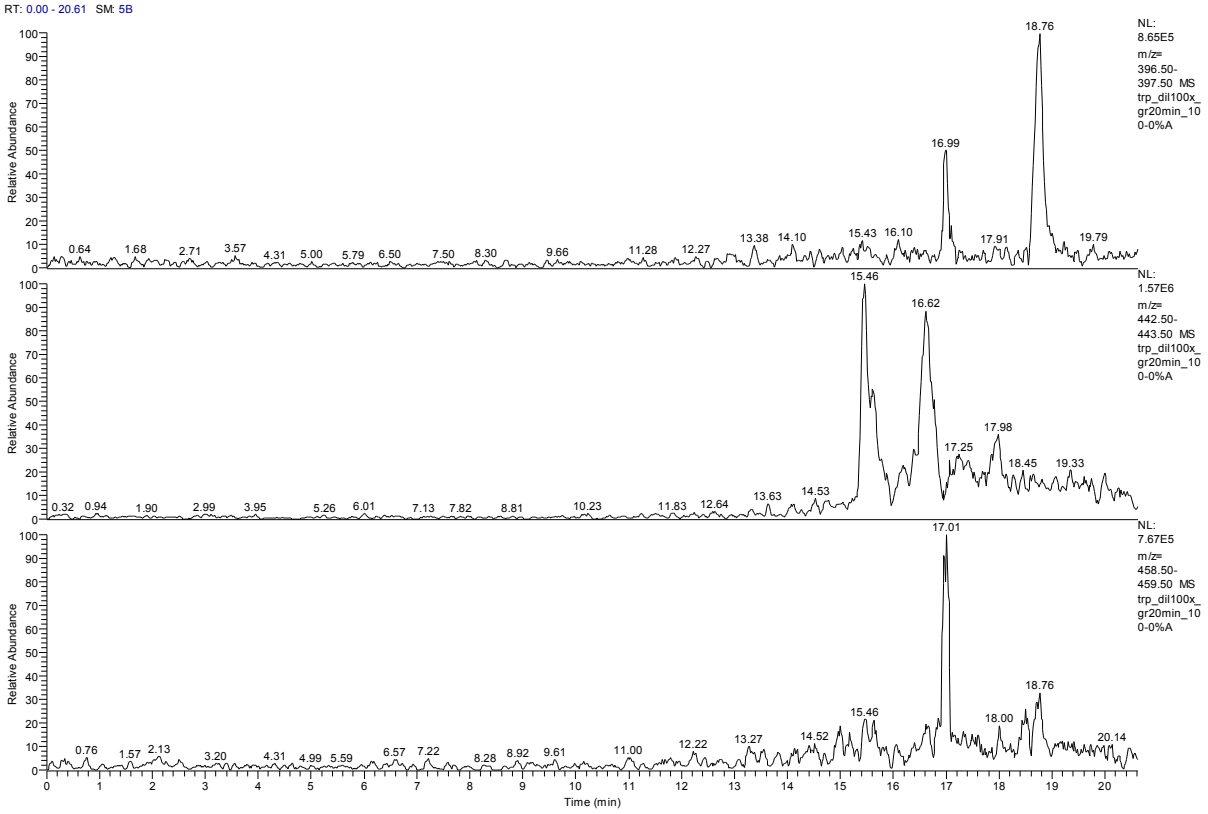


Conclusions

1. Amount of the created dimer (m/z 519) is average considering the other amino acids.
2. There were three suggestions of adduct formation between tryptophane and Lim-1-OOH. The m/z were as follows: 411, 413 and 369. Neither of them was confirmed.

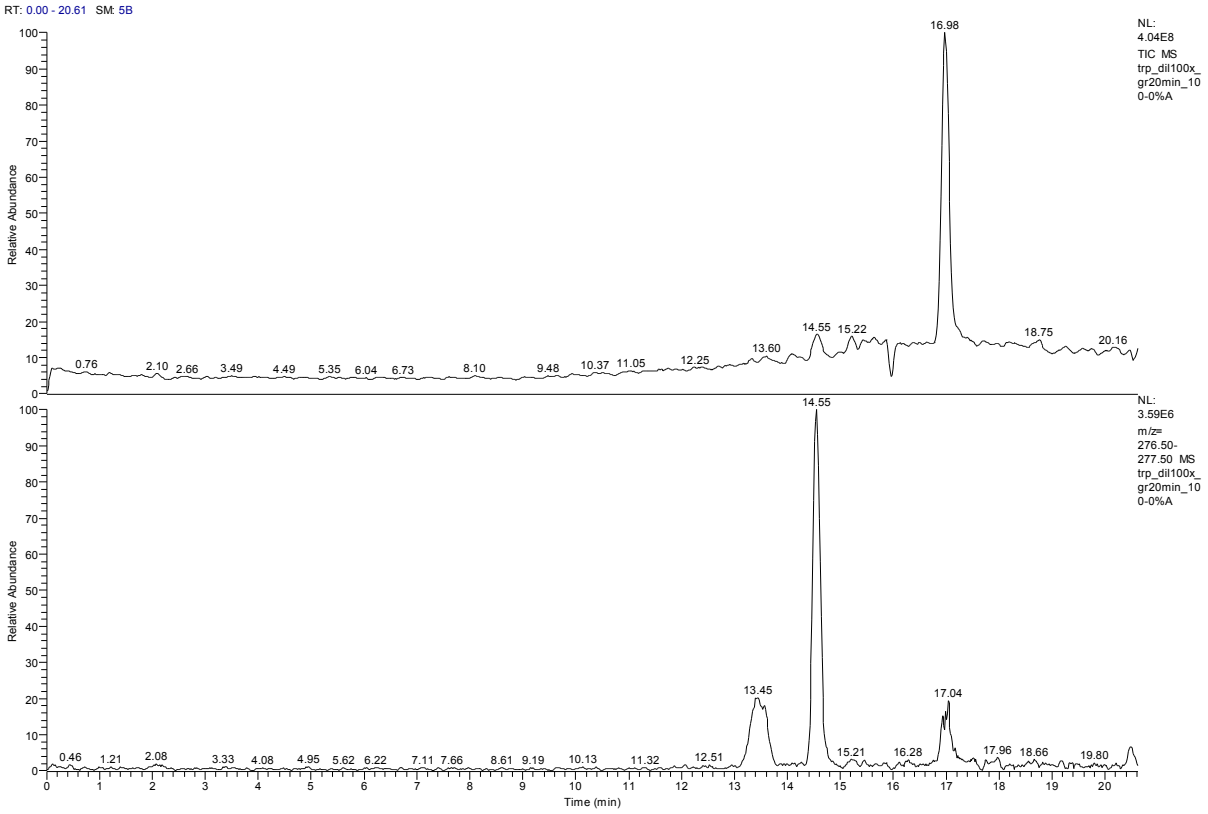
3. The protonated masses 397 and 443 could be possibly adducts, as they gave relatively straight narrow peaks. Nevertheless their intensity in RICs was too low to make them being of relevant interest (*see Tab.5.*).
4. The 459 protonated mass also showed relatively straight narrow peak with very low intensity. We did not focus on the structure of the previous m/z (397 and 443 respectively). On the contrary we assume the 459 protonated mass being a fragment of tryptophane dimer (519 – loss of acetyl) rather than an adduct even though the fragment is created in a very low amount.

On the top: RIC of m/z 397, in the middle: RIC of m/z 443, at the bottom: RIC of m/z 459.



5. The protonated mass 277 showed nice peak and was of relatively high intensity. Considering the masses of the protected tryptophane and the Lim-1-OOH, a compound with the mass 276 could not be adduct of these two. Therefore the protonated mass 277 is rather considered to be an oxidated protected tryptophane (261 plus one oxygen).

On the top: TIC of tryptophane reaction mixture diluted 100x, at the bottom: RIC of m/z 277

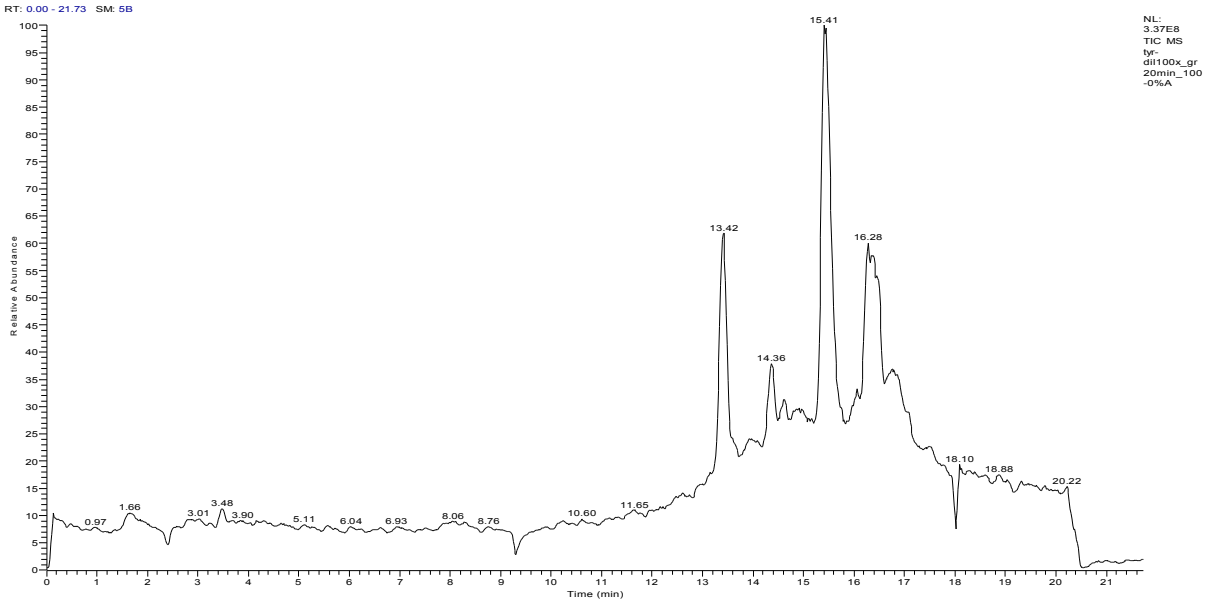


→ **Lim-1-OOH is not considered to create any likely adducts with tryptophane.**

8.6. Tyrosine

Results of the tyrosine reaction mixture diluted 100 times were collected. The screening experiments were performed on LCQ DECA. The intensity of TIC was 3.37 E8 counts. The intensities of particular protonated masses are summarized in Tab.6. On the chromatogram were seen several clearly separated narrow peaks:

TIC of the tyrosine reaction mixture diluted 100x



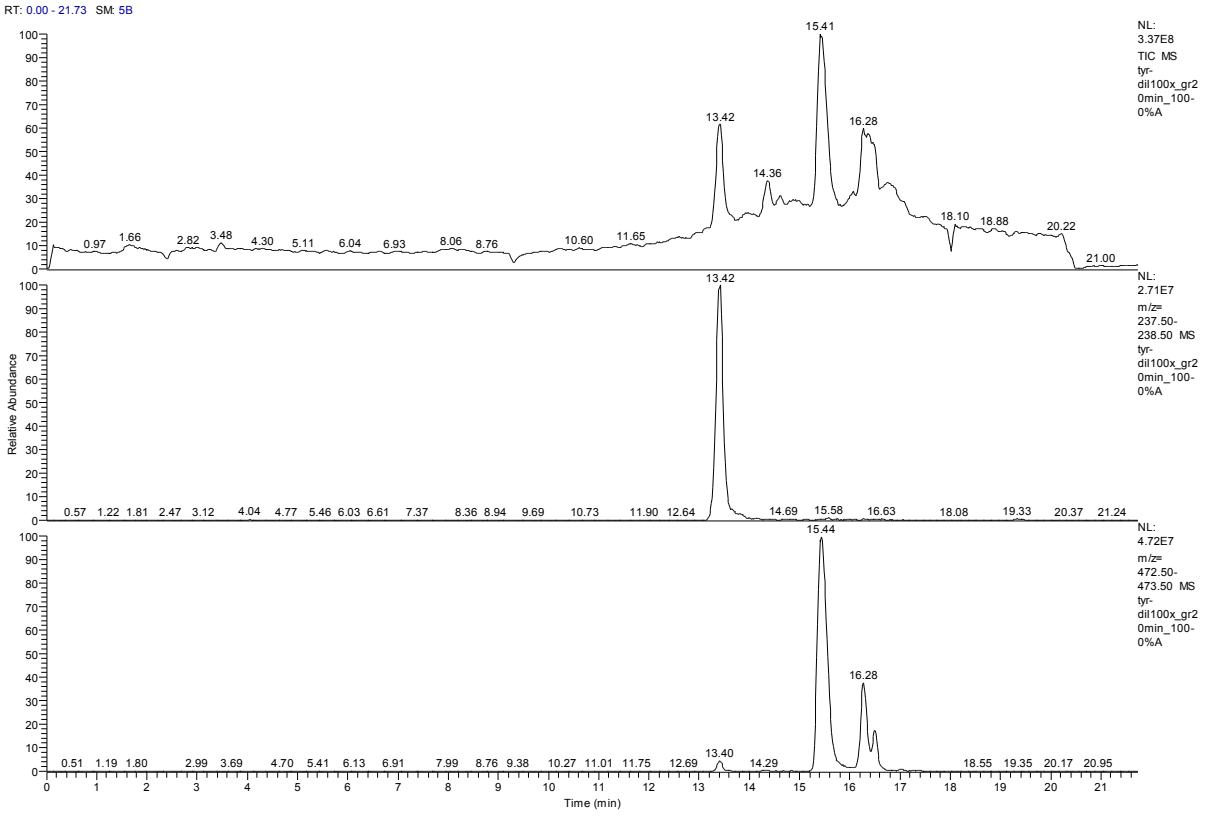
The peak at **RT 13.42 min** belongs to m/z 238 (tyr), at **14.36 min** m/z 459 was a base peak and also m/z 371 was observed, at **RT 15.41 min** m/z 473 (dimer) together with lower m/z 371 was seen, and at **16.39 min** peak of m/z 708 was the base peak.

Tab.6. Intensities of RICs in tyrosine reaction mixture diluted 100x

Protonated mass	*Intensity in RIC*	*Note*
238	**2.71 E7**	**protected tyr**
473	**4.72 E7**	**dimer of tyr**
406 Tyr + Lim-1-OOH + H^+	2.50 E5	
404 Tyr + Lim-1-OOH – 2H + H^+	2.12 E5	
422 Tyr + Lim-1-OOH plus O + H^+	2.65 E5	
420 Tyr + Lim-1-OOH plus O – 2H + H^+	2.29 E5	
438 Tyr + Lim-1-OOH plus 2 O + H^+	2.65 E5	
436 Tyr + Lim-1-OOH plus 2 O – 2H + H^+	2.37 E5	
388 Tyr + Lim-1-OOH minus O – 2H + H^+	4.52 E5	estimated adduct from University of Gothenburg.
390 Tyr + Lim-1-OOH minus O + H	3.68 E5	
374 Tyr + Lim-1-OOH minus 2 O + H^+	3.96 E5	
372 Tyr + Lim-1-OOH minus 2 O – 2H + H^+	**6.22 E6**	✓ high intensity only in RIC
459	**7.86 E5**	✓ high intensity
708	**1.50 E7**	✓ high intensity

N.B. ✓ stands for relatively straight narrow peak, **possible adduct**

On the top: TIC of tyrosine reaction mixture diluted 100x, in the middle: RIC of m/z 238 (tyr), at the bottom: RIC of m/z 473 (dimer)

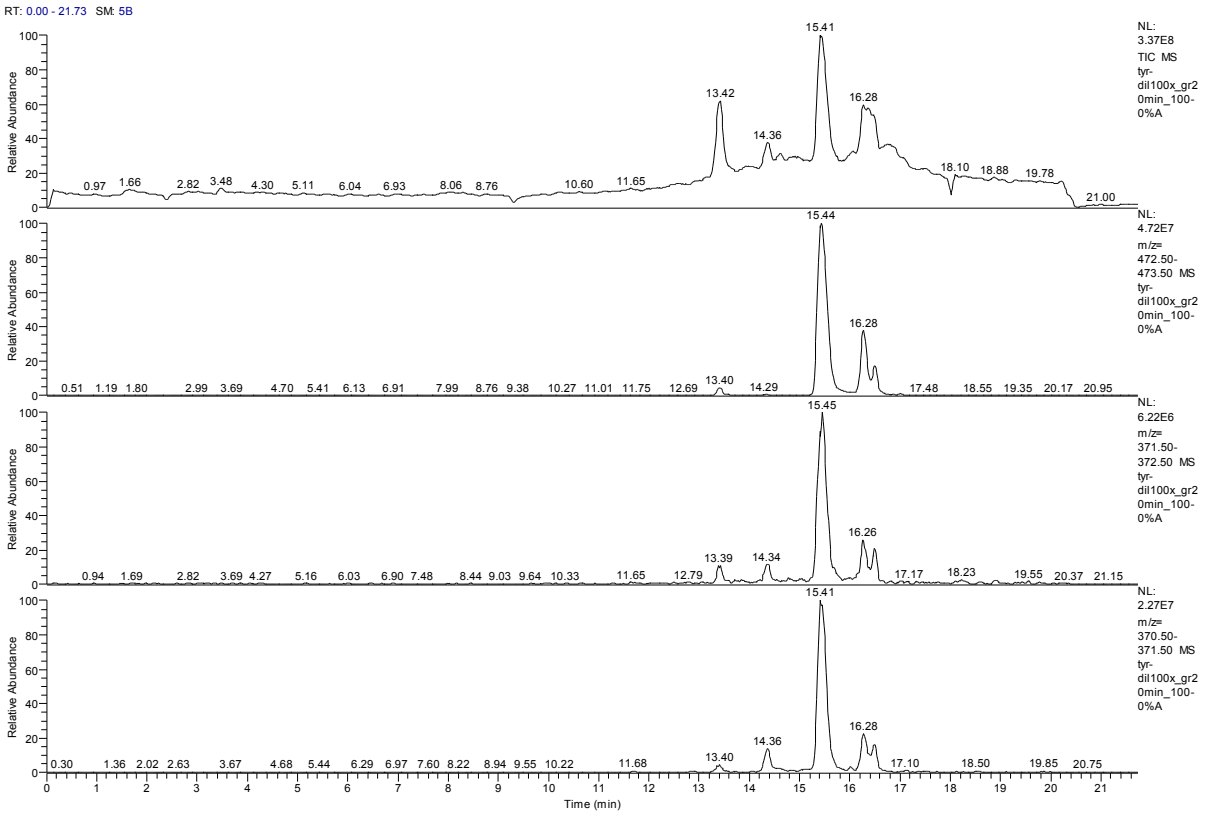


Conclusions

1. Tyrosine formed the highest amount of dimer (m/z 473) from all the amino acids. Tyrosine is prone to polymerization, which is in accordance of the Lim-2-OOH experiments. Moreover, tyrosine showed to form also trimer (236 + 235 + 236 + H^+ = 708).
2. From University of Gothenburg came a suggestion of one formed adduct of m/z 388, which was however not confirmed.

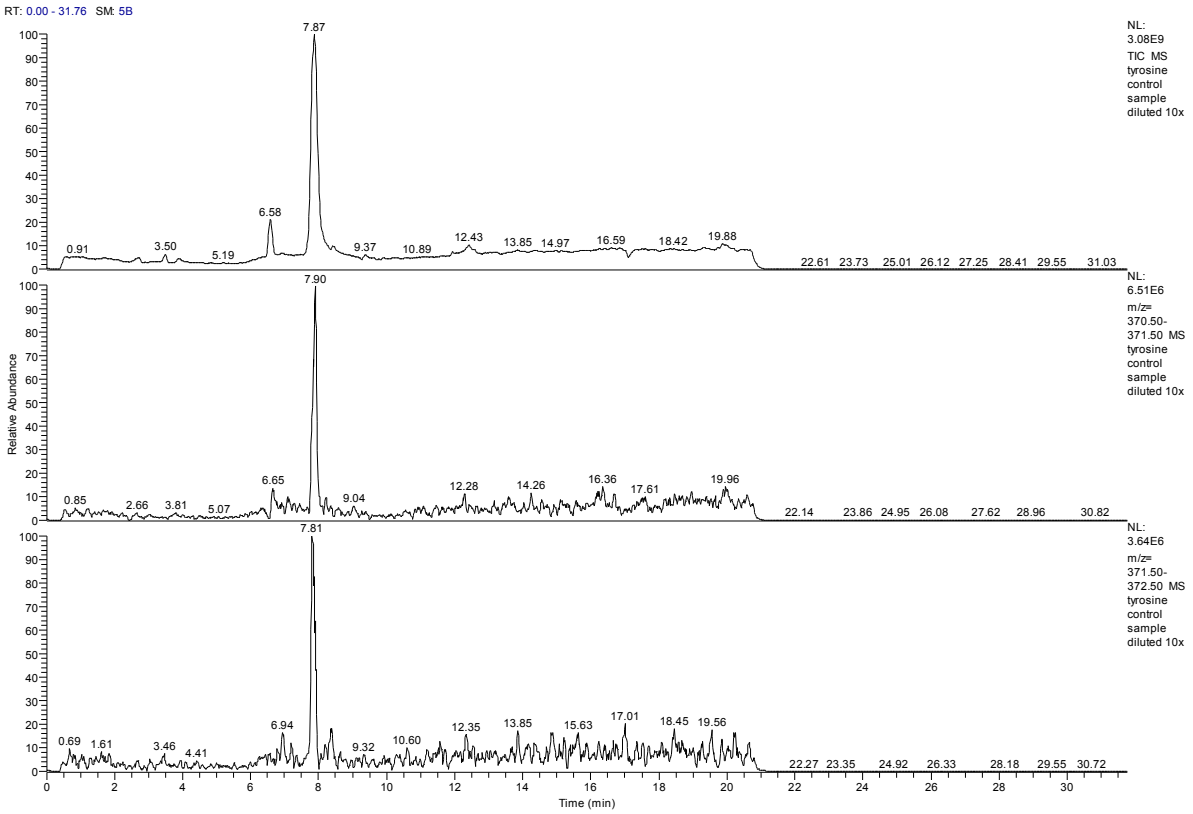
3. The protonated mass 372 showed a relatively nice intense peak in RIC. In the mass spectra the protonated mass 371 was often seen in the company of the tyrosine dimer (m/z 473) and trimer (m/z 708) respectively. We found out a correlation between the protonated masses 372 and 371, as they had the same position in the chromatograms. The m/z 371 from the two of them was more intense (371: 2.27 E7 x 372: 6.22 E6 counts). Moreover, both were in the same position as the tyrosine dimer.

On the top: TIC of the reaction mixture of tyrosine diluted 100x, second position: RIC of m/z 473 (dimer), third position: RIC of m/z 372, at the bottom: RIC of m/z 371



The 371 protonated mass was also later during the MS^n experiments revealed to be a fragment of tyrosine dimer. Fragments of m/z 473 tyrosine dimer: 371.02, 413.06, 441.04, 431.16. Both of the protonated masses were also observed during the control sample experiment.

The tyrosine control sample diluted 100x: on the top: TIC, in the middle: RIC of m/z 371, at the bottom: RIC of m/z 372



Neither 371, nor 372 protonated mass is therefore considered as a Lim-1-OOH adduct.

4. The protonated mass 459 was also in the MS^n experiments confirmed to be a fragment of tyrosine dimer, as it gave the same fragments as the dimer itself. M/z 459: 371.11, 413.08, 441.05, 430.88 and m/z 473: 371.02, 413.06, 441.04, 431.16.

→ **The control sample experiment had to be performed to see, whether the protonated masses 371 and 372 are present as well.**

→ **The MS^n experiments revealed later the structure of the protonated masses 459 and 708.**

→ **However from what was already written, Lim-1-OOH is not considered to create adducts with tyrosine**

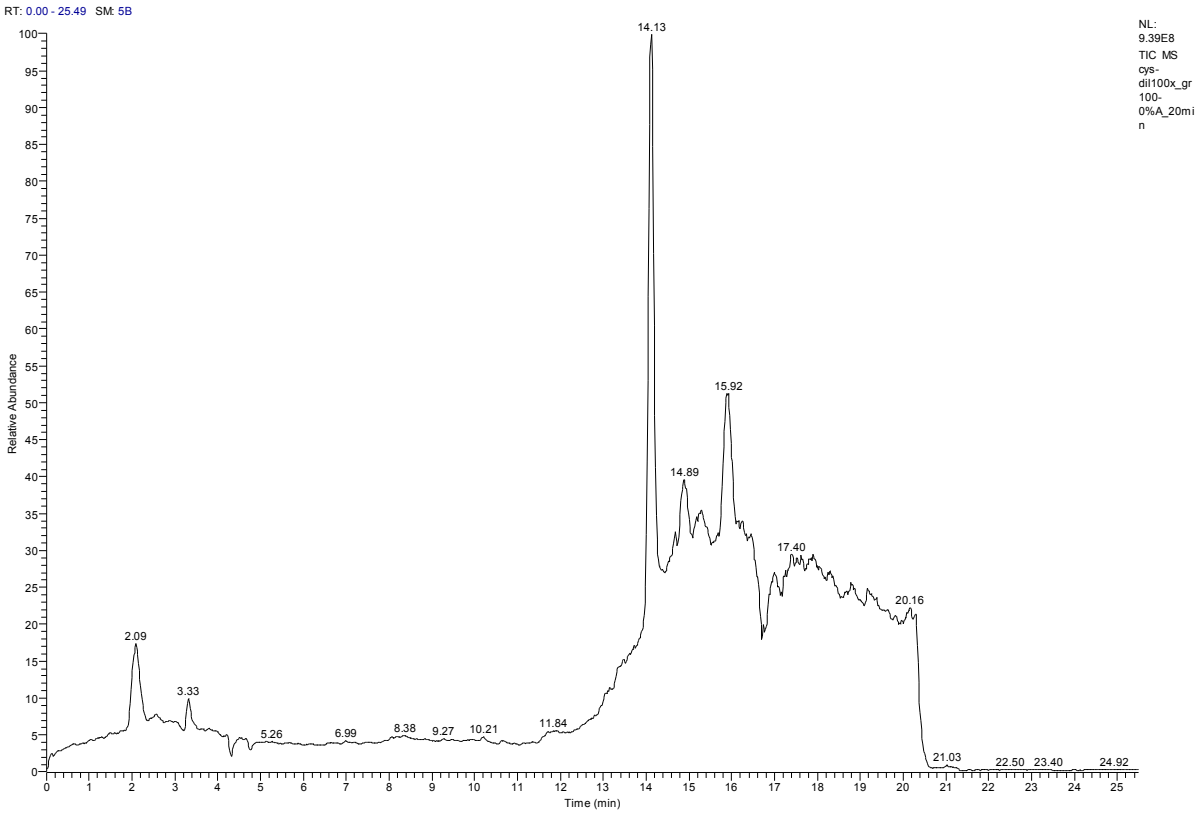
8.7. Cysteine

Cysteine was expected to create adducts with Lim-1-OOH via a radical pathway as the results of the Lim-2-OOH experiment were taken into account. It was the cysteine that bound to that hydroperoxide.

The reaction mixtures were analysed on both instruments and the samples were diluted 10 and 100 times. The intensities of TIC were as follows: LCQ DECA diluted 10x: 2.92 E9, LCQ DECA diluted 100x: 9.39 E8 and TSQ Vantage diluted 100x: 5.24 E7 counts.

The masses which were focused on after the screening experiments were divided into three groups A, B and C. Unfortunately several discrepancies were met, as the two instruments were implied in the experiment. Slightly different results regarded the m/z 362, 348, 346, 330 and 328 (group B). Group A was consisted of m/z, that were suspected from being adducts (680, 636, 521, 503, 339 and 312). The last group C is created by the protonated masses, that gave straight peaks of relatively high intensity, but which were not considered as adducts (220, 234). The overview of the m/z and their intensities are summarized in Tab.7.

LCQ DECA: TIC of the cysteine reaction mixture diluted 100x



The main separated peaks gave the following protonated masses as the base peaks: at **RT 14.13 min** m/z 353 (cys dimer), at **RT 14.89 min** m/z 521 and at **15.92 min** m/z 680 and 503.

On the RICs the protonated masses 636 (**RT 15.92 min**), 339 (**RT 11.84 min**), 312 (**RT 13.93 min**), 220 (**RT 13.04 min**) and 234 (**RT 10.06 min**) showed straight narrow peaks. What regards the protonated masses 362, 348, 346, 330 and 328 respectively, occurrence of their peaks differed on both instruments. When TSQ Vantage was implied, rather multiplied peaks were observed.

Tab.7. Intensities of RICs in cysteine reaction mixture

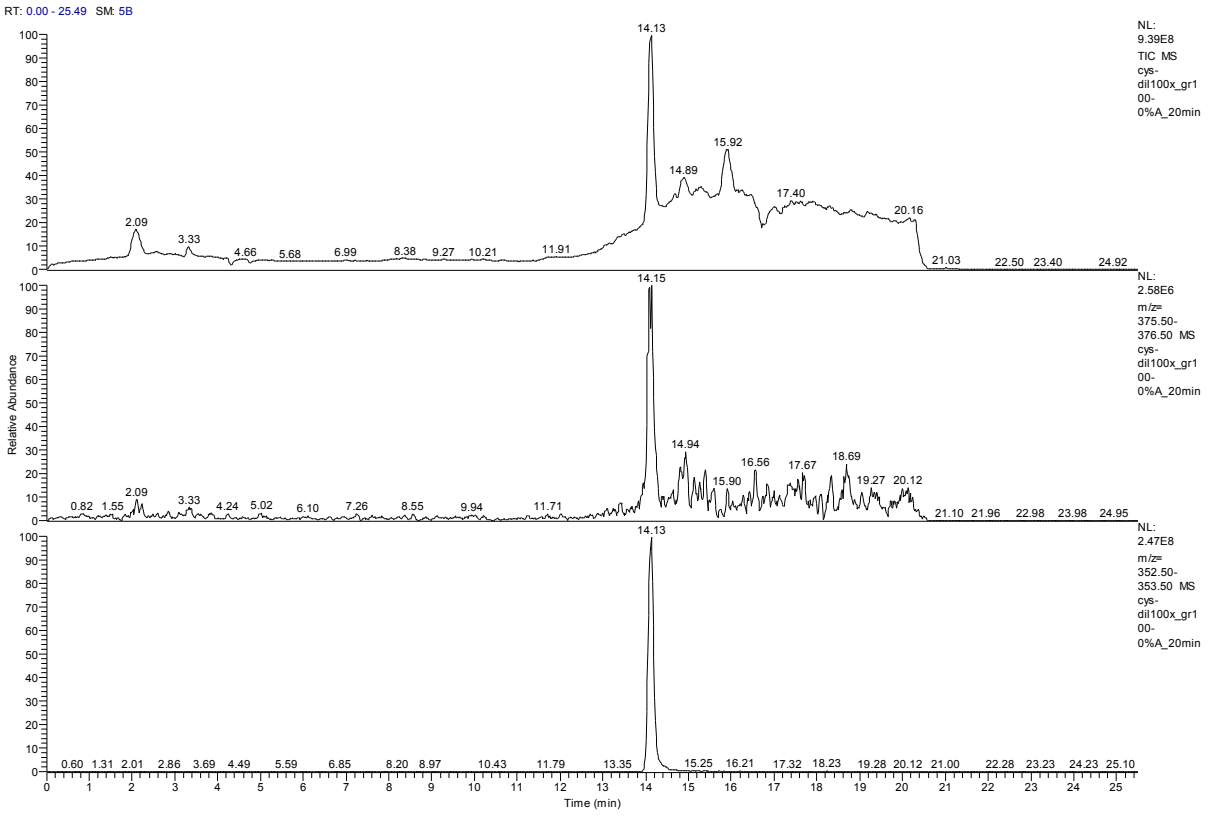
Protonated mass	*Intensity in RIC LCQ DECA dil.10x*	*Intensity in RIC LCQ DECA dil. 100x*	*Intensity in RIC TSQ Vantage dil. 100x*	*Note*
178	**1,21 E8**	**1,23 E7**	**4,72 E5**	**protected cys**
353	**9,30 E8**	**2,48 E8**	**1,49 E7**	**dimer of cys**
346 Cys + Lim-1-OOH + H^+	**4,23 E6**	**6,73 E5**	**1,15 E5** more peaks	✓
344 Cys + Lim-1-OOH – 2H + H^+	8,30 E6	1,27 E6	8,75 E4	
362 Cys + Lim-1-OOH plus O + H^+	**4,23 E7**	**2,08 E6**	**1,20 E5** more peaks	✓
360 Cys + Lim-1-OOH plus O – 2H + H^+	1,05 E7	1,34 E6	2,39 E5	
378 Cys + Lim-1-OOH plus 2 O + H^+	4,80 E6	9,23 E5	7,93 E4	
376 Cys + Lim-1-OOH plus 2 O – 2H + H^+	**2,58 E6**	**2,58 E6**	**7,16 E5**	**Na^+ add. of cys dimer.**
330 Cys + Lim-1-OOH minus O + H^+	**1,23 E7**	**2,29 E6**	**2,24 E5** more peaks	✓
328 Cys + Lim-1-OOH minus O – 2H + H^+	**1,18 E7**	**1,24 E6**	**1,70 E5** more peaks	✓
314 Cys + Lim-1-OOH minus 2 O + H^+	6,67 E6	1,05 E6	2,16 E5	
312 Cys + Lim-1-OOH minus 2 O – 2H + H^+	**3,26 E7**	**4,49 E6**	**2,89 E6**	✓
348	**9,73 E6**	**6,50 E5**	**1,12 E5** more peaks	✓
680	**2,51 E8**	**3,33 E7**	**6,03 E5**	✓
503	**1,19 E8**	**1,63 E7**	**4,77 E5**	✓
521	**3,46 E7**	**4,71 E6**	**8,58 E4**	✓
636	**3,70 E7**	**3,63 E6**	**1,87 E5**	✓
339	**1,33 E8**	**2,77 E6**	**4,04 E6**	✓
220	**2,43 E8**	**4,17 E6**	**2,20 E5**	
234	**7,60 E7**	**2,01 E6**	**1,16 E6**	

N.B. ✓ stands for relatively straight narrow peak, **possible adduct**

Conclusions

1. Amount of the cysteine dimer (m/z 353) was over average among the other amino acids. The 376 protonated mass gave a narrow straight peak. The sodium adduct of cysteine dimer is of m/z 375. The 376 has the same position and is of less intensity. Thus being in narrow correlation and is not considered as an Lim-1-OOH adduct.

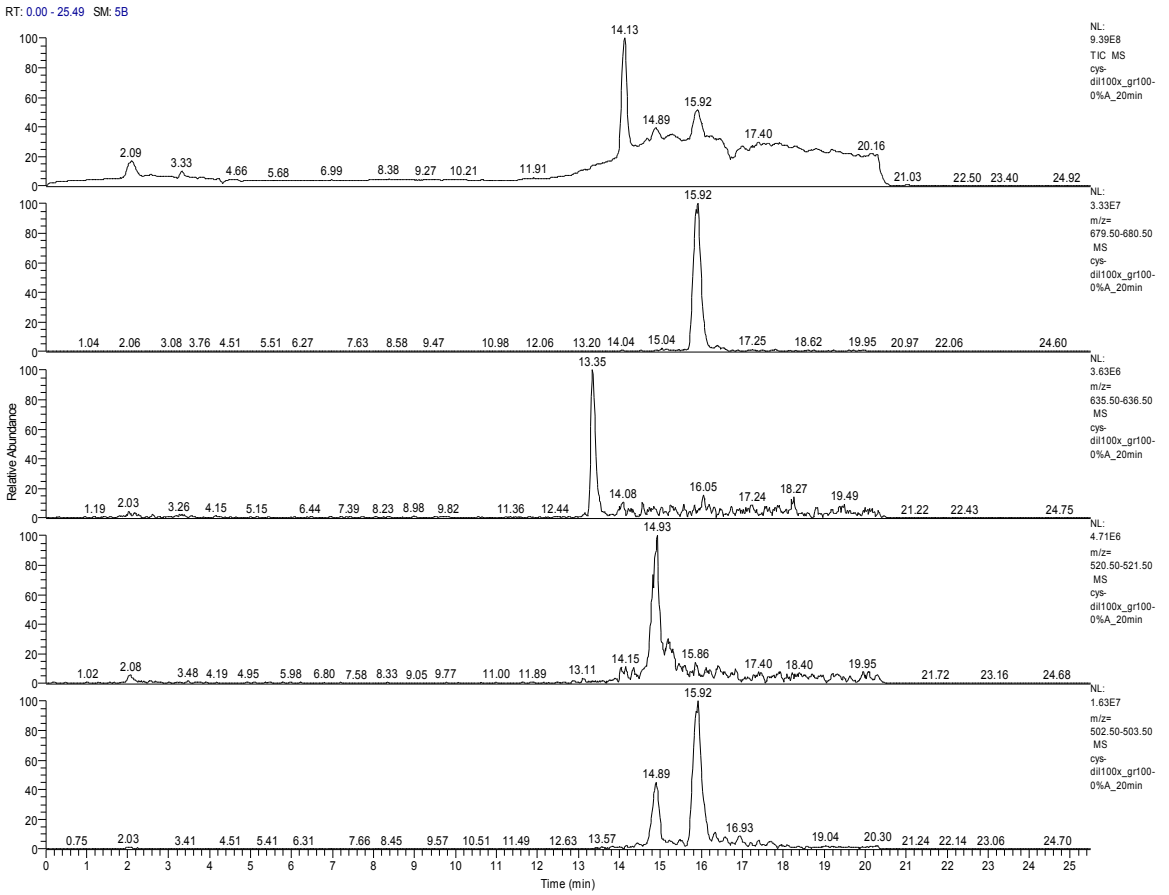
LCQ DECA: on the top: TIC of the cysteine reaction mixture diluted 100x, in the middle: RIC of m/z 376, at the bottom: RIC of m/z 353 (dimer of cys)



2. There were no suggestions from the University of Gothenburg about the masses of potential adducts. Even though the consideration of adduct formation was eligible.

3. The protonated masses 680, 636, 521, 503, 339 and 312 were considered to be adducts of Lim-1-OOH.

LCQ DECA: on the top: TIC of the cysteine reaction mixture diluted 100x, on second position: RIC of m/z 680, on the third position RIC of m/z 636, on the fourth position: RIC of m/z 521, at the bottom RIC of m/z 503

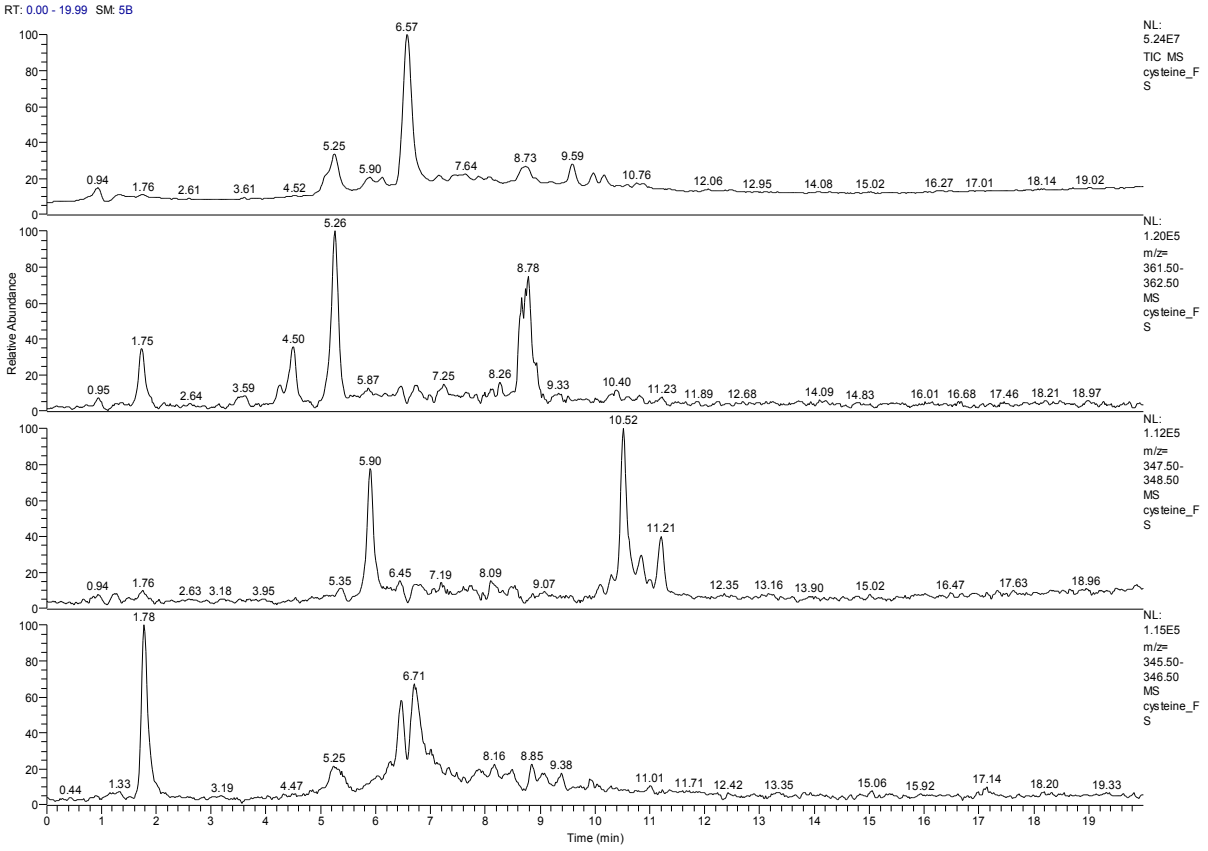


4. The two protonated masses of 220 and 234 gave straight peaks of relatively high intensity, however when considering the masses of protected cysteine (177) and Lim-1-OOH (168), they cannot be adducts of the two compounds. (Results not shown.)

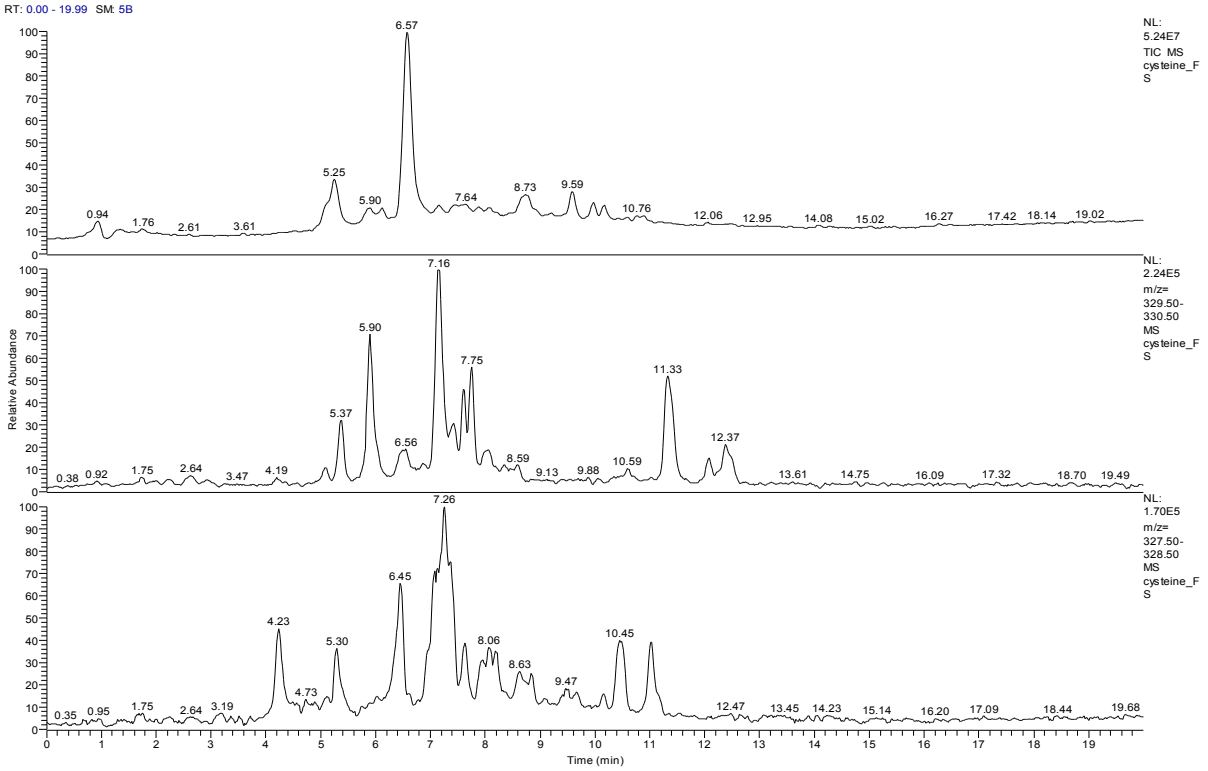
5. The protonated masses 362, 348, 346, 330 and 328 gave somewhat confusing results, as they differ according to which instrument was used or which dilution had been applied. Nevertheless they might be very well formed adducts and we remained focused on those.

   When the experiment was carried out on the TSQ Vantage instrument, all the masses gave multiplied peaks no matter what the dilution was (10x or 100x). *See chromatograms below*.

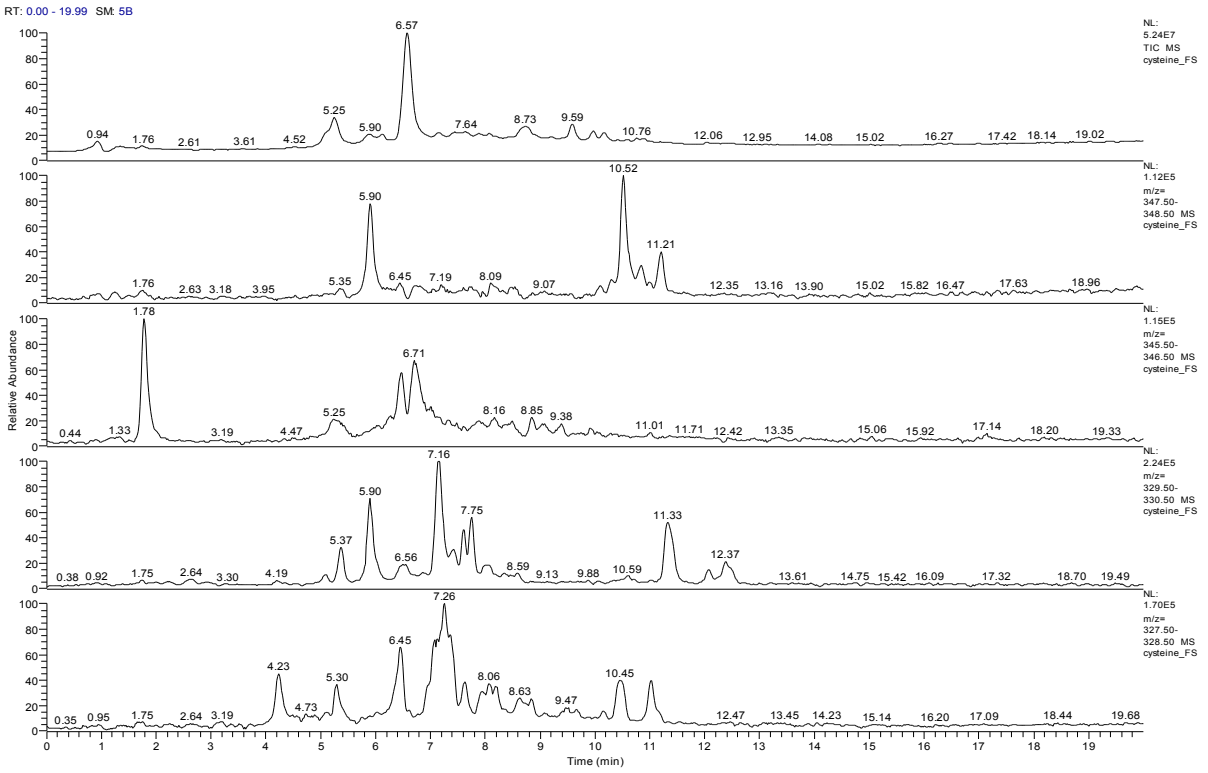
TSQ Vantage: on the top: TIC of cysteine reaction mixture diluted 10x, on the second position: RIC of m/z 362, on the third position RIC of m/z 348, at the bottom: RIC of m/z 346



TSQ Vantage: on the top: TIC of cysteine reaction mixture diluted 10x, in the middle: RIC of m/z 330, at the bottom: RIC of m/z 328

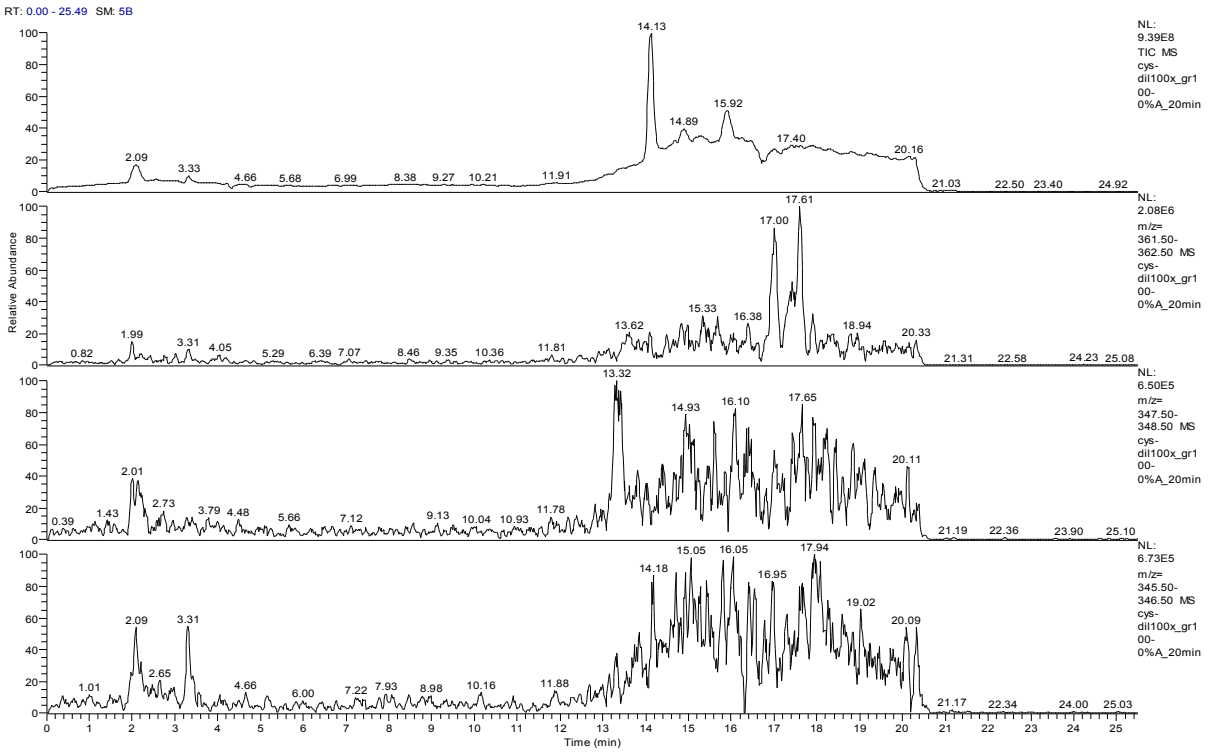


TSQ Vantage: on the top: TIC of cysteine reaction mixture diluted 100x, on the second position: RIC of m/z 348, on the third position RIC of m/z 346, on the fourth position: RIC of m/z 330, at the bottom: RIC of m/z 328

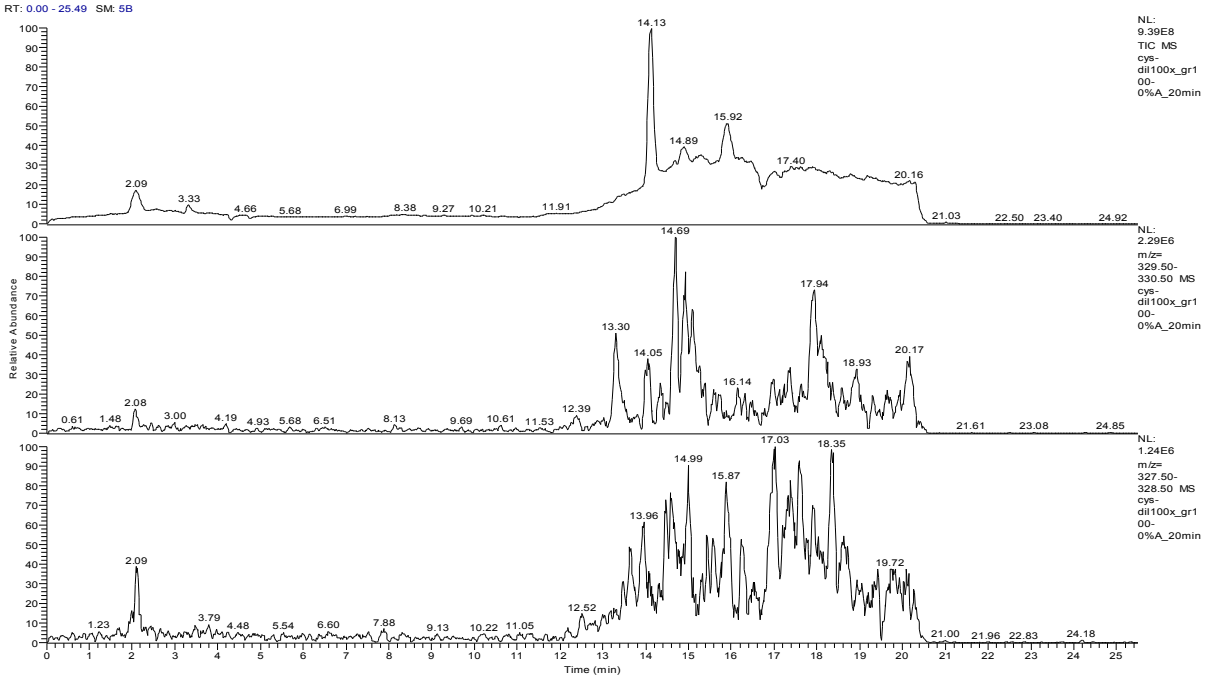


On the contrary on LCQ DECA instrument in 100x diluted sample no nice peaks of the mentioned protonated masses were observed.

LCQ DECA: on the top: reaction mixture of cysteine diluted 100x, on the second position: RIC of m/z 362, on the third position: RIC of m/z 348, at the bottom: RIC of m/z 346

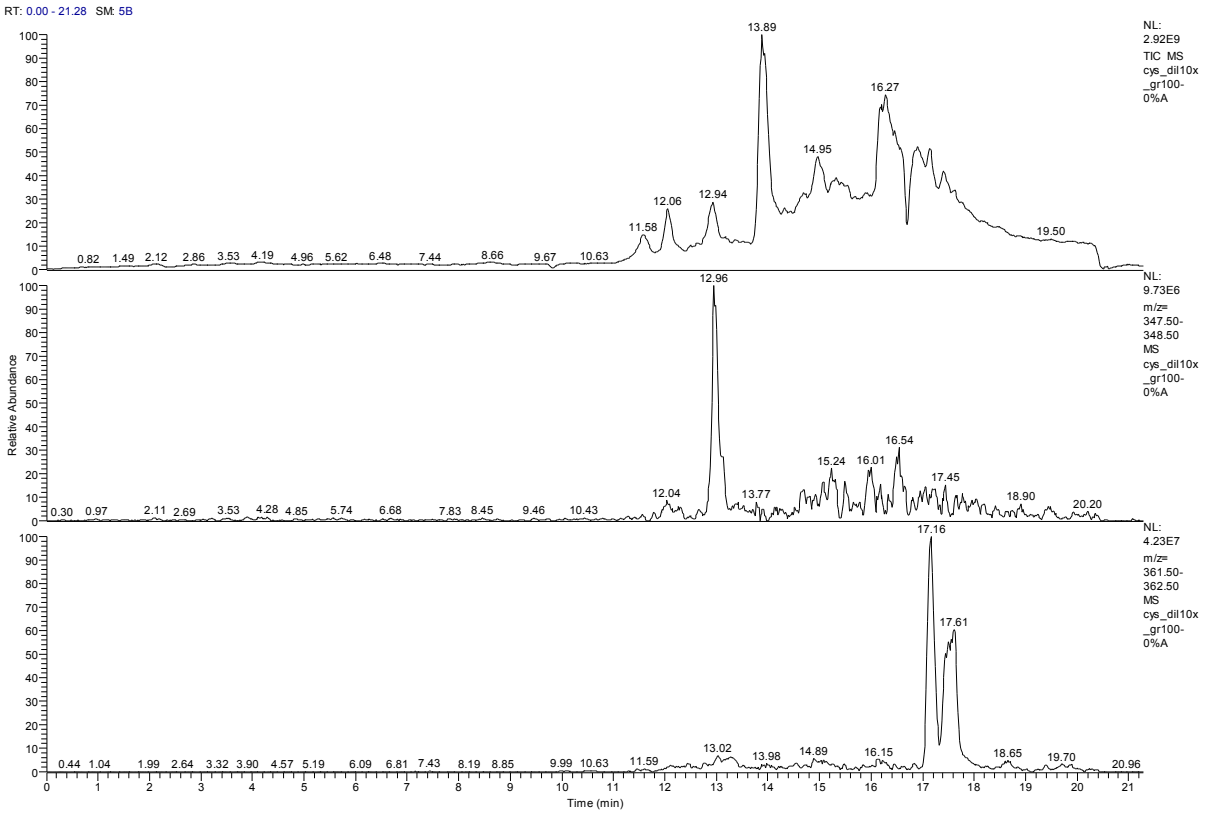


LCQ DECA: on the top: reaction mixture of cysteine diluted 100x, in the middle: RIC of m/z 330, at the bottom: RIC of m/z 328



Similarly looked all the masses also when the sample was diluted only 10x. The exceptions were the protonated masses 362 and 348.

LCQ DECA: on the top: reaction mixture of cysteine diluted 10x, in the middle: RIC of m/z 348, at the bottom: RIC of m/z 362.



→ **The control sample experiments had to be performed to eliminate the protonated masses that are not relevant adducts.**

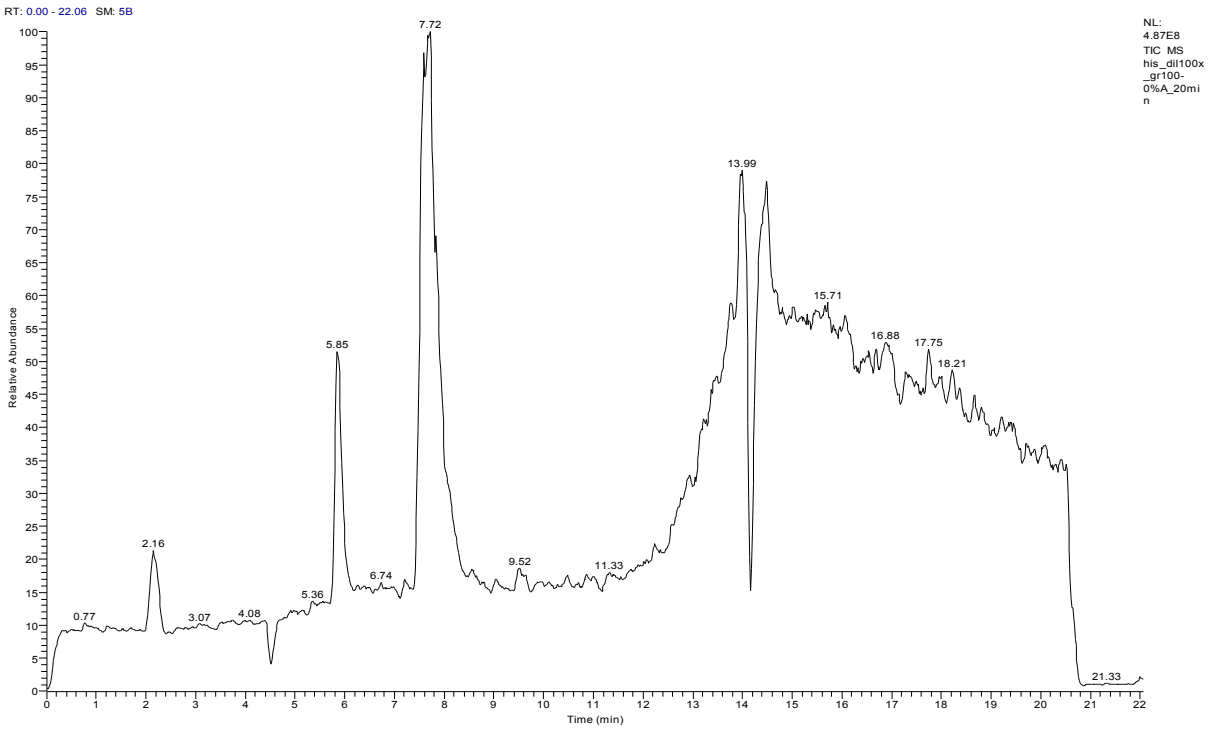
→ **The MSn experiment had to be carried out to get more detailed information about the possible adducts formation.**

→ **In this stage of the project cysteine was strongly suspected to form adducts with Lim-1-OOH (A: 680, 636, 521, 503, 339, 312), (B: 362, 348, 346, 330, 328), (C: 220, 234).**

8.8. Histidine

The reaction mixture of histidine was analysed on both instruments. The sample was diluted 100 times. On the contrary to the cysteine, the results were this time comparable and their resume is included in Tab.8. The intensities of TIC were: LCQ DECA: 4.87 E8 and TSQ Vantage: 2.52 E7 counts. (The chromatograms depicted in the following text come only from the LCQ DECA instrument.)

TIC of histidine reaction mixture diluted 100x



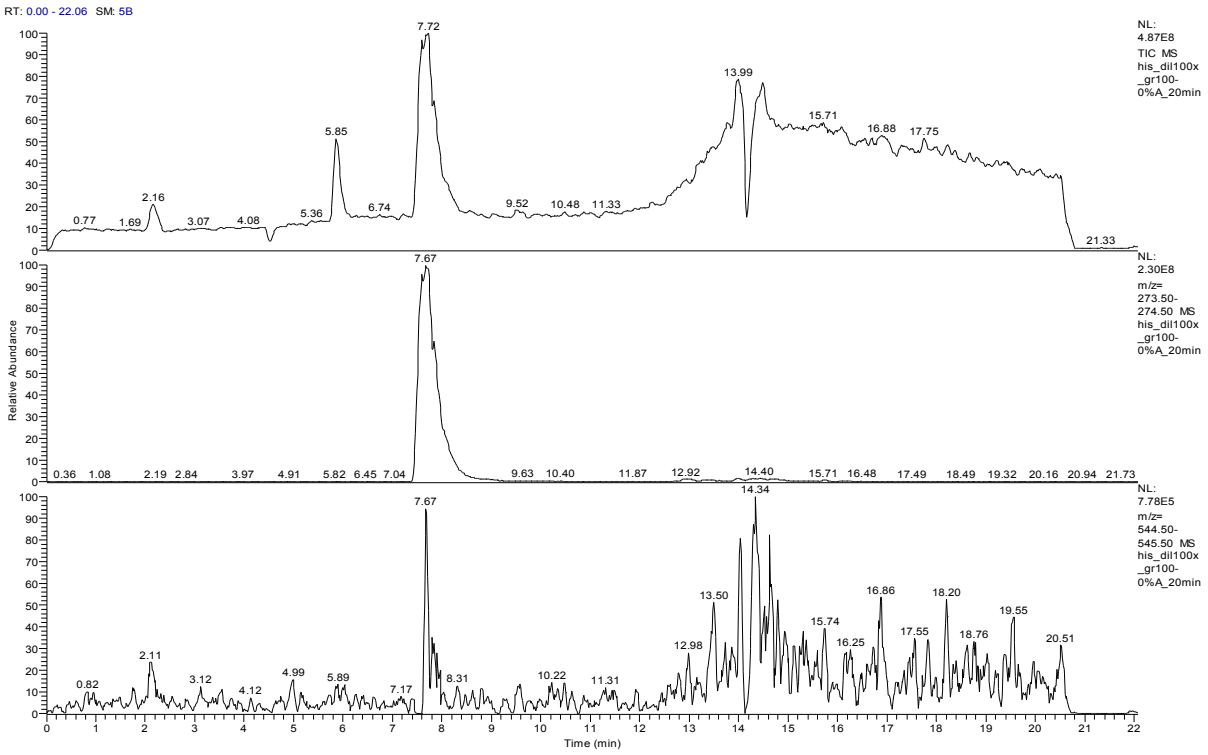
The chromatogram showed several separated peaks. At the **RT 5.86 min** the base peak of m/z 260 was observed. At **7.67 min** the protonated mass 274, which corresponds to the protected histidine was the base peak. At **13.97 min** it was the protonated mass 442 and at **14.44 min** the protonated mass 428. The m/z 426, which was suggested as adduct by the University of Gothenburg, showed three different peaks at **RT 13.35, 14.47 and 15.54 min**. The last m/z we focused on (410) gave peak **at RT 14.48 min**.

Tab.8. Intensities of RICs in histidine reaction mixture

Protonated mass	*Intensity in RIC LCQ DECA*	*Intensity in RIC TSQ Vantage*	*Note*
274	**2,30 E8**	**1,12 E7**	**protected his**
545	**7,78 E5**	**4,97 E4**	**dimer of his**
442 His + Lim-1-OOH + H^+	**4,87 E7**	**1,94 E6**	✓
440 His + Lim-1-OOH – 2H + H^+	8,27 E6	3,67 E5	
458 His + Lim-1-OOH plus O + H^+	7,48 E6	5,82 E4	
456 His + Lim-1-OOH plus O – 2H + H^+	2,08 E6	4,29 E4	
474 His + Lim-1-OOH plus 2 O + H^+	1,44 E6	3,69 E4	
472 His + Lim-1-OOH plus 2 O – 2H + H^+	1,17 E6	9,25 E3	
426 His + Lim-1-OOH minus O + H^+	**6,23 E6**	**4,26 E4**	✓
424 His + Lim-1-OOH minus O – 2H + H^+	3,13 E6	8,03 E4	
410 His + Lim-1-OOH minus 2 O + H^+	**3,91 E7**	**1,61 E6**	✓
408 His + Lim-1-OOH minus 2 O – 2H + H^+	5,60 E5	1,47 E4	
428	**1,12 E7**	**4,36 E5**	✓
260	**9,23 E7**	**1,00 E7**	relatively narrow intense peak

N.B. ✓ stands for relatively straight narrow peak, high intensity, **possible adduct**

On the top: TIC of the reaction mixture of his diluted 100x, in the middle: RIC of m/z 274 (his), below: RIC of m/z 545 (dimer of his).

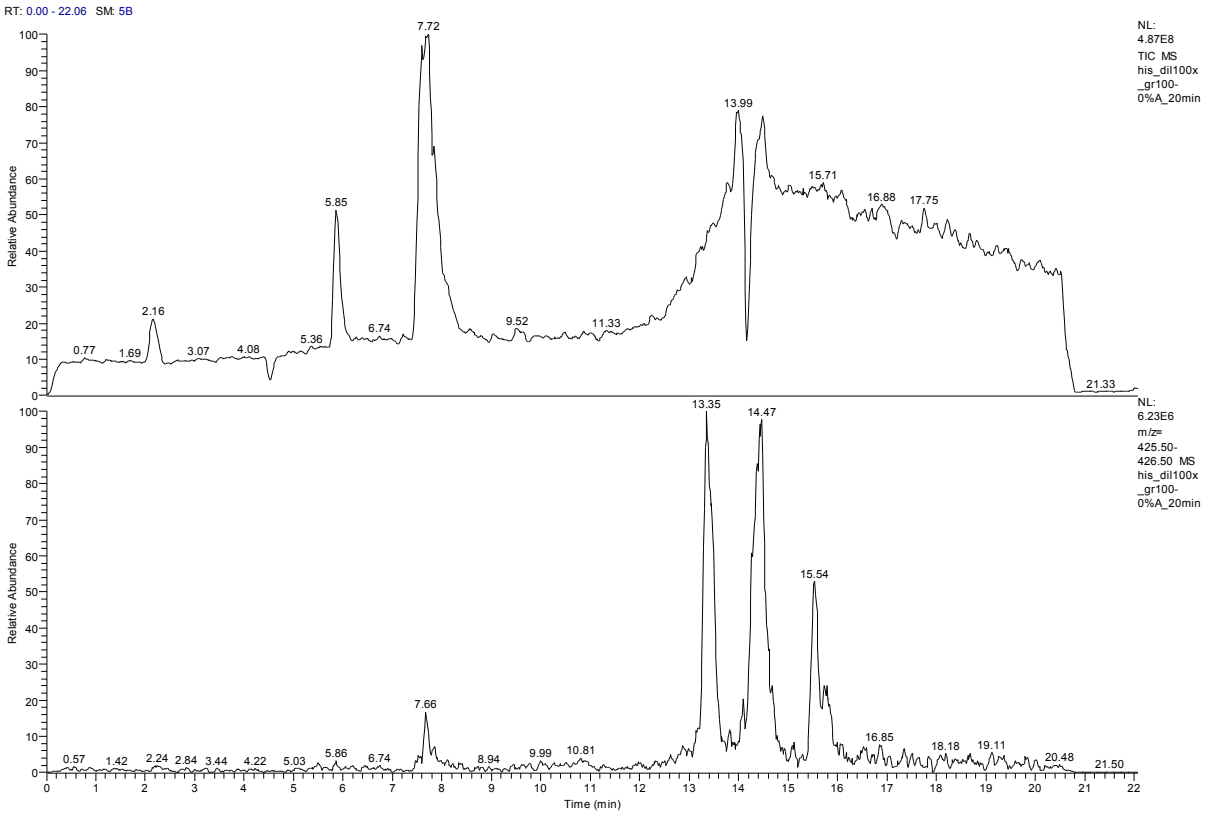


Conclusions

1. Amount of the created dimer was under averaged considering the other amino acids.

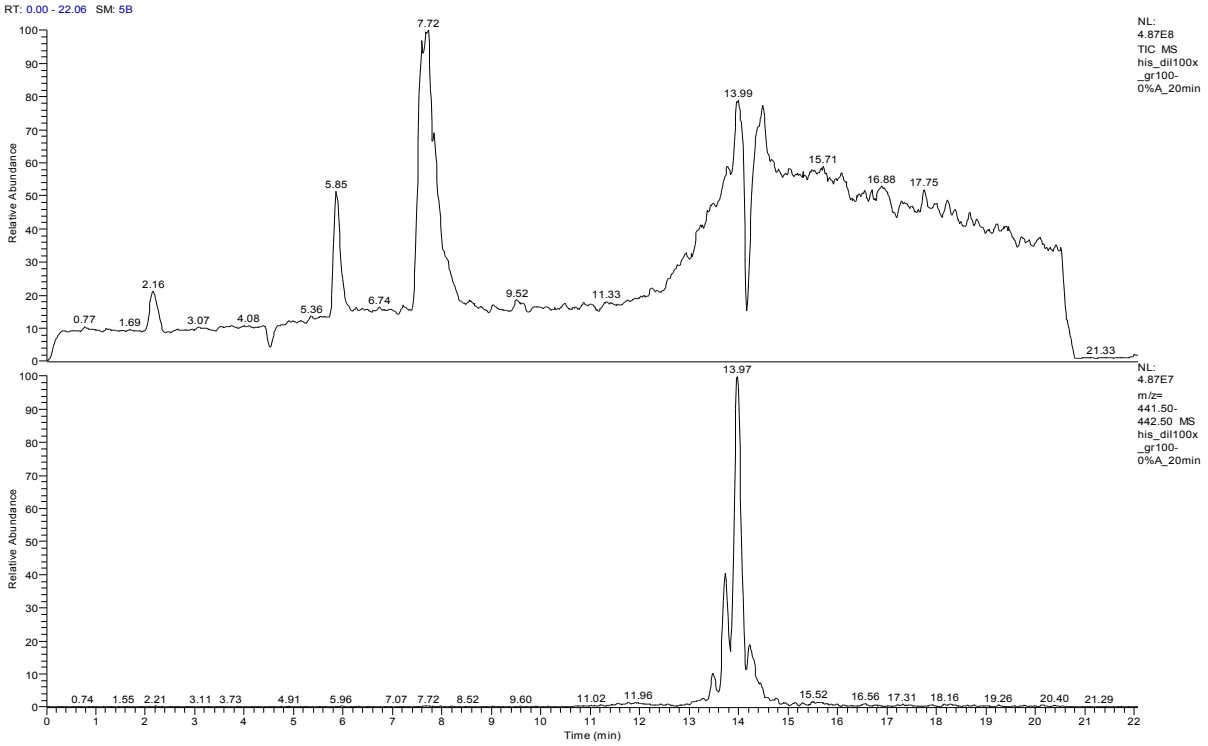
2. The University of Gothenburg suggested one possible adduct with m/z 426, which was indeed observed.

On the top: TIC of the reaction mixture of histidine diluted 100x, at the bottom: RIC of m/z 426



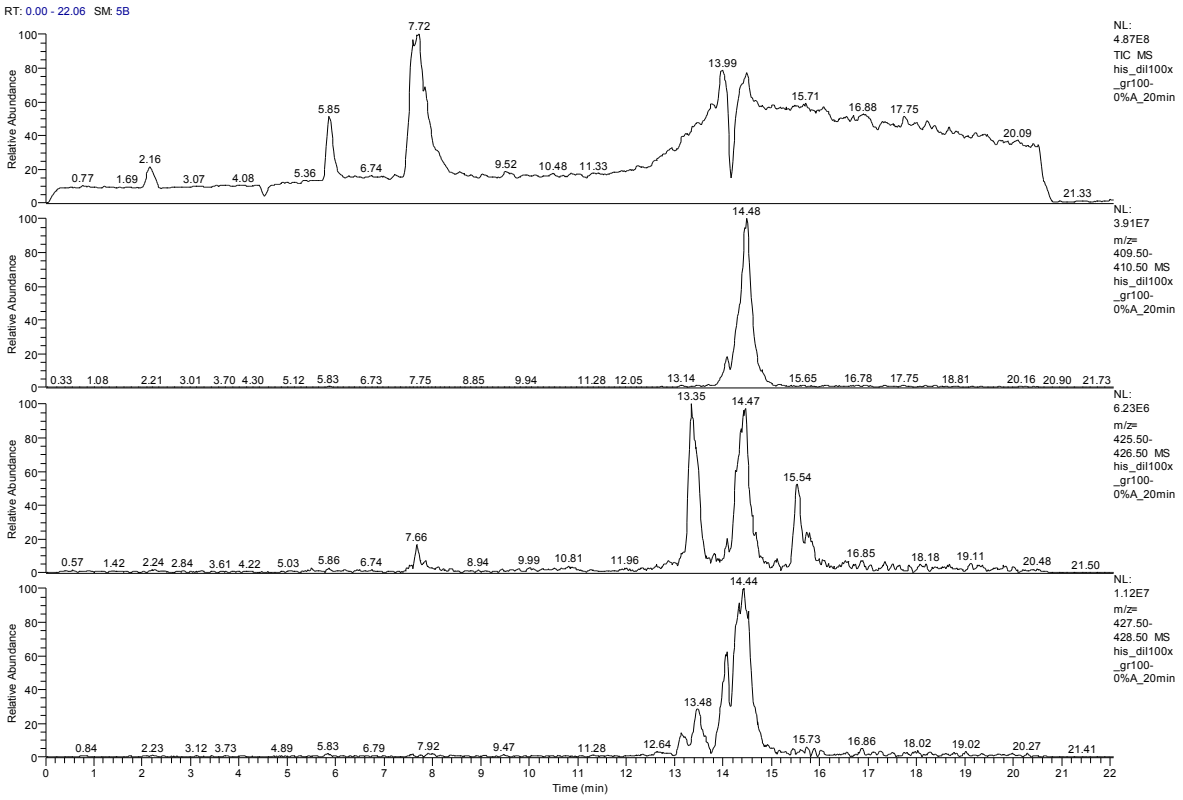
3. The protonated masses 442, 428 and 410 respectively were moreover considered to be another formed adducts.

On the top: TIC of the reaction mixture of histidine diluted 100x, at the bottom: RIC of m/z 442



There were correlations found among the positions of the m/z 428, 426 and 410. The m/z 410 was therefore considered rather being a fragment of 428 (loss of water).

On the top: TIC of the reaction mixture of histidine diluted 100x, on the second position: RIC of m/z 410, on the third position: RIC of m/z 428, at the bottom: RIC of m/z 428.



4. The 260 protonated mass was considered being rather a fragment of histidine. When assuming the masses of the protected histidine and the Lim-1-OOH, a compound with m/z 260 could not be adduct anyway.

→ **The control sample experiments were carried out to find out, whether the m/z might be relevant as adducts.**

→ **The MS^n experiments had to be performed for being more informed about the possible adducts.**

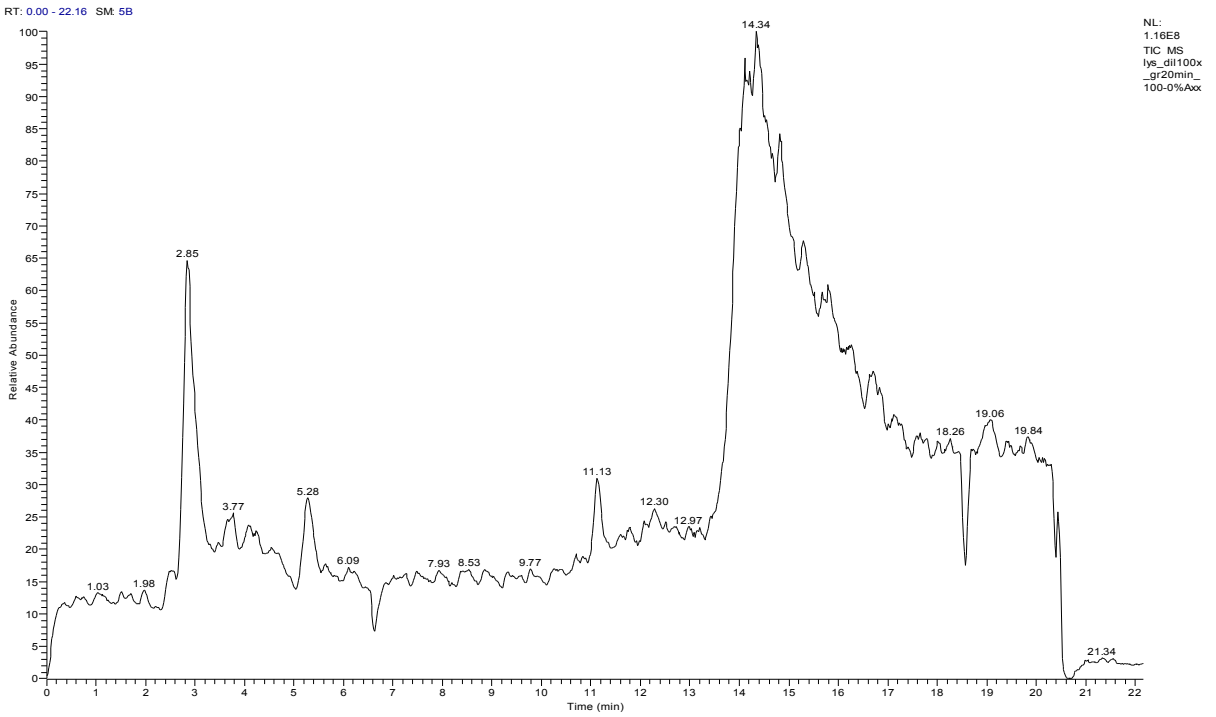
→ **Histidine was after the first stage of the project suspected to create adducts with Lim-1-OOH. The masses being focused in the following stages were: 442, 428, 426 and 410.**

8.9. Lysine

The lysine reaction mixture was analysed on both instruments. The samples were diluted 100 times and the resume of the results is shown in Tab.9. TIC intensities were: LCQ DECA 1.16 E8 and TSQ Vantage 2.52 E7 counts. The results gained from the two instruments are comparable with only two slight exceptions regarding the protonated masses 339 and 353.

On the chromatogram there are several well separated peaks, even though the last one is relatively broad and comprised several interesting m/z:

LCQ DECA : TIC of lysine reaction mixture diluted 100x



The peak at **RT 2.85 min** belongs to the protected lysine (m/z 203). The following peak at **RT 5.28 min** showed two intense m/z: 301 and 243 the latter one being the base peak. The **RT 11.13 min** belongs to the 444 protonated mass.

Moreover m/z of 273 (**RT 3.68 min**), 371 (**RT 4.04 min**), 287 (**RT 4.58 min**), 321 (**RT 14.34 min**), 411 (**RT 14.55 min**), 353 (**RT 14.55 min**), 339 (**RT 15.83 min**) and 311 (showed multiplied peaks) with relatively straight narrow peaks were seen. All of these protonated masses were focused in following stage of the control sample experiment, to determine, which of the m/z could belong to the adducts of lysine and Lim-1-OOH.

Tab.9. Intensities of RICs in lysine reaction mixture

Protonated mass	*Intensity in RIC LCQ DECA*	*Intensity in RIC TSQ Vantage*	*Note*
203	**3,17 E7**	**1,67 E7**	**protected lys**
403	3,96 E5	3,06 E4	Dimer of lys
371 Lys + Lim-1-OOH + H^+	**1,30 E6**	**8,51 E5**	**possible adduct**
369 Lys + Lim-1-OOH – 2H + H^+	7,12 E5	5,61 E5	
387 Lys + Lim-1-OOH plus O + H^+	3,50 E5	9,05 E4	
385 Lys + Lim-1-OOH plus O – 2H + H^+	2,55 E5	1,41 E5	
403 Lys + Lim-1-OOH plus 2 O + H^+	3,96 E5	3,06 E4	
401 Lys + Lim-1-OOH plus 2 O – 2H + H^+	2,41 E5	6,61 E4	
355 Lys + Lim-1-OOH minus O + H^+	4,97 E5	4,19 E4	estimated adduct from the University of Gothenburg
353 Lys + Lim-1-OOH minus O – 2H + H^+	**2,20 E6**	**3,08 E5**	✓
339 Lys + Lim-1-OOH minus 2 O + H^+	**1,12 E6**	**8,61 E4**	✓
337 Lys + Lim-1-OOH minus 2 O – 2H + H^+	4,55 E5	2,47 E4	
444	**3,49 E6**	**1,79 E6**	✓
411	**1,49 E6**	**5,05 E4**	✓
321	**2,81 E6**	**6,61 E4**	✓
311	**4,88 E6**	**1,44 E6**	✓
301	**4,69 E6**	**1,34 E7**	✓
243	**6.52 E6**	**7,68 E6**	✓
273	**7,17 E6**	**1,29 E7**	✓
287	**8.56 E6**	**5,25 E6**	✓

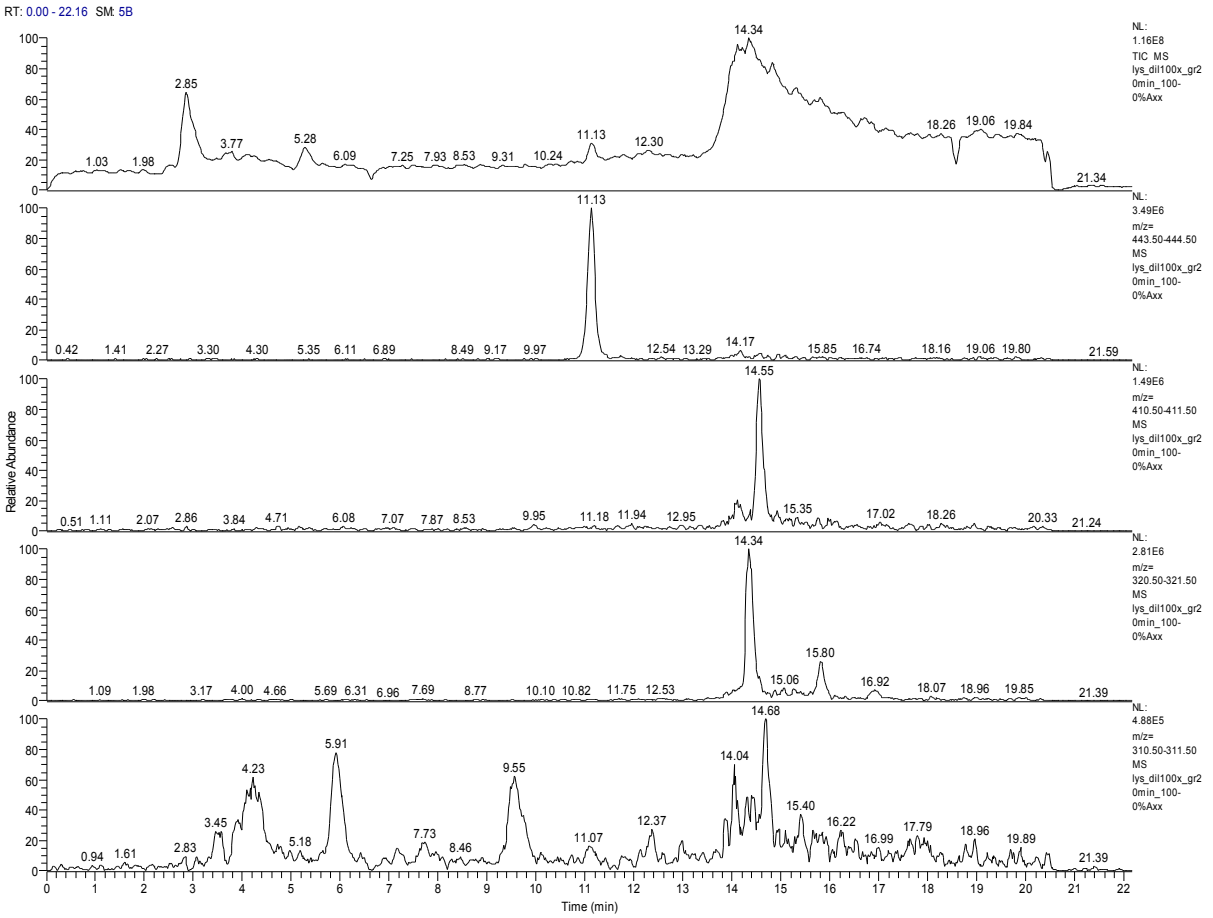
N.B. ✓ stands for relatively straight narrow peak, high intensity, possible adduct

Conclusions

1. Lysine created the least amount of the dimer (403). It can be concluded, that lysine does not create any dimer.
2. From the University of Gothenburg came one suggestion of adduct formation of m/z 355. However such adduct was not confirmed.

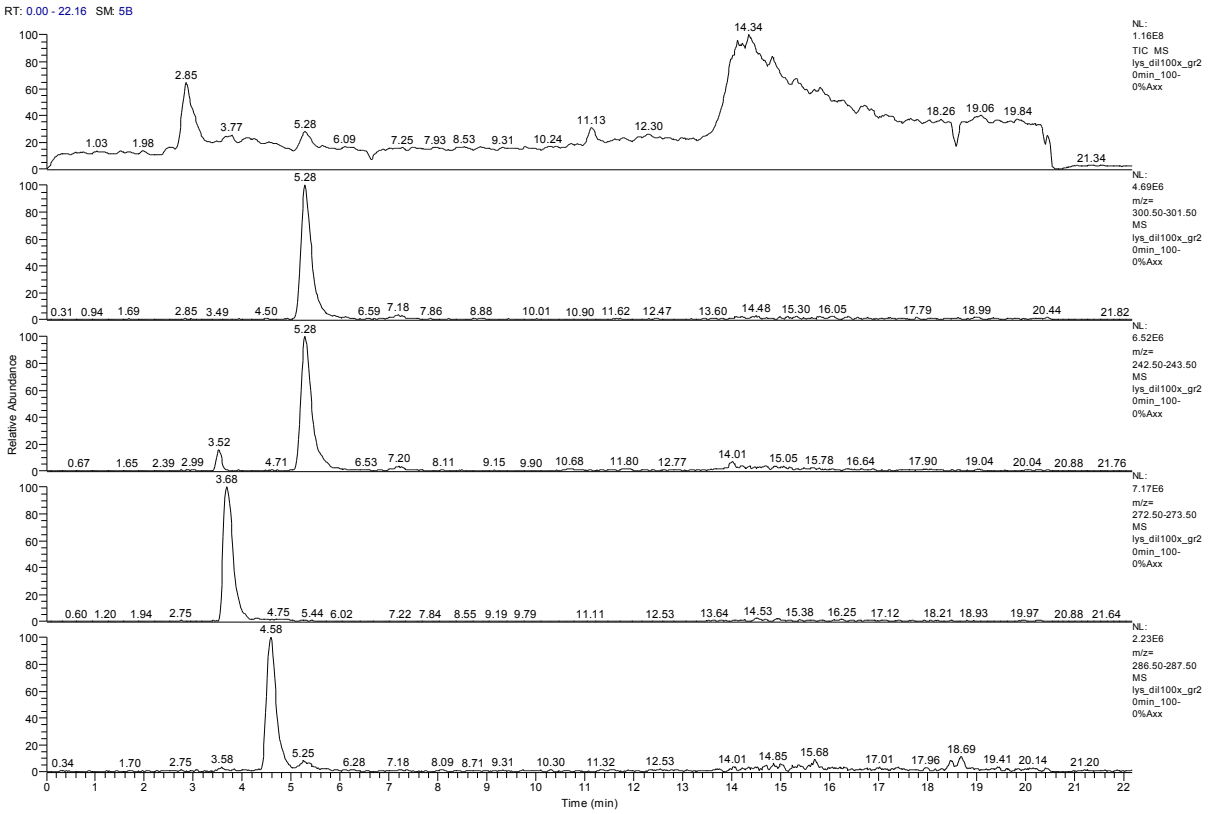
3. The protonated masses 444, 411, 321, 371, 353 and 339 were suspected to be possible adducts of Lim-1-OOH.

LCQ DECA: on the top: TIC of the lysine reaction mixture diluted 100x, on the second position: RIC of m/z 444, on the third position: RIC of m/z 411, on the fourth position: RIC of m/z 321, at the bottom: RIC of m/z 311 (not considered as an adduct)



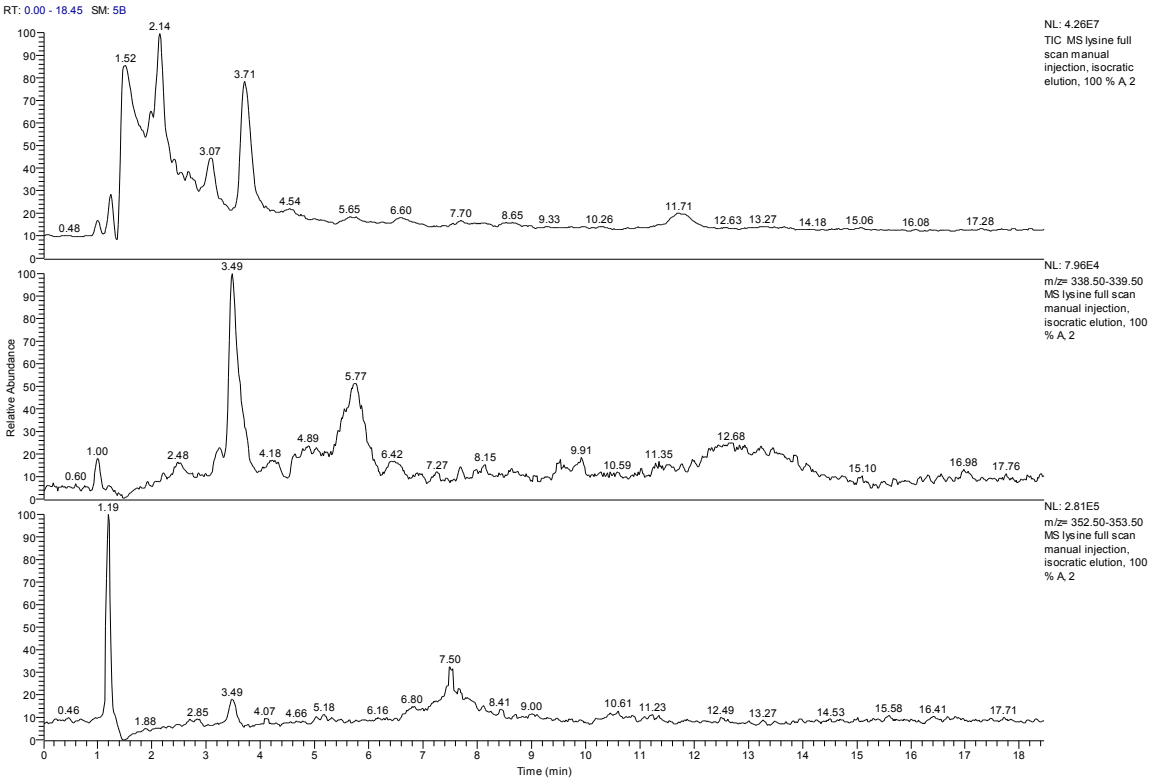
4. Also m/z 311, 301, 287, 273 and 243 showed nice and relatively intensive peaks in the RIC, however when considering the masses of lysine (202) and Lim-1-OOH (168), they do not correspond with the potential adducts. Nevertheless they remained within the range of interest during the following control sample experiments.

LCQ DECA: on the top: TIC of the lysine reaction mixture diluted 100x, on the second position: RIC of m/z 301, on the third position: RIC of m/z 243, on the fourth position: RIC of m/z 273, at the bottom: RIC of m/z 287



5. The 339 and 353 protonated masses were better seen on LCQ DECA instrument. On Vantage the intensity was relatively lower, though still quite narrow peaks were observed (*see the Tab.9. and the chromatogram below*).

TSQ Vantage: On the top: TIC of the lysine reaction mixture diluted 100x, in the middle: RIC of m/z 339, at the bottom: RIC of m/z 353. (Note the separation on TIC is different because of the isocratic elution!).



→ **Control sample experiments had to be performed after the first stage of the project to distinguish, which m/z could belong to relevant adducts.**

→ **The MS^n experiments were planned for getting more information about the potential adducts.**

→ **After the screening experiments the Lim-1-OOH is considered to form adducts with lysine. (M/z: 444, 411, 371, 321, 353, 339) and (311, 301, 287, 273, 243).**

9. Control samples experiments

9.1. Overview of the preliminary results I

The first step in the project included the screening experiments based on LC/MS analyses of the reaction mixtures of Lim-1-OOH and amino acids in the presence of the Fe (III) porphyrin complex. Certain m/z were focused on. They were either those suggested by the University of Gothenburg as possible adducts from radical reactions, or those calculated and estimated and at last m/z of relatively high intensities and/or showing sharp narrow peak.

What regards **alanine, leucine** and **tryptophane**, it could be stated already after performing the screening experiments, that none of them creates adducts with Lim-1-OOH. Tyrosine formed huge amount of the dimer. **Tyrosine** was not expect to form any adducts with the hydroperoxide, however two protonated masses (**371** and **372**) were suspected pf being formed adducts. Therefore tyrosine was involved in the next stage of the project, too.

Cysteine was on the contrary strongly suspected from adducts formation, as in the previous experiment with Lim-2-OOH (*ref. 1*) it was the only amino acid to form relevant amount of the adducts. After the screening experiments following protonated masses remained focused: **680, 636, 521, 503, 362, 348, 346, 330, 328, 312, 339, 220** and **234**.

Histidine was expected to create adducts, too. And indeed, several protonated masses were found and remained focused in the next project‘s stages. They were: **442, 428, 426** and **410**.

Lysine was less probable to bind to Lim-1-OOH; nevertheless it was the third amino acid to show possible adducts formation. The aimed protonated masses were: **444, 411, 321, 371, 353, 339, 311, 301, 287, 273** and **243**.

9.2. Aim of the control samples experiments

The control samples experiments should have eliminated those protonated masses, which did not correspond to adducts of amino acids and Lim-1-OOH formed via a radical

pathway. Each control sample consisted of the amino acid and Lim-1-OOH. The radical initiator was not used. Once the protonated mass was observed also in the control sample, it was not considered as adduct. Adducts of biological relevance between Lim-1-OOH and amino acids are considered to be formed only via the radical mechanism and not in reaction mixture without the radical initiator.

9.3. Preparation of the control samples

The control samples experiments were performed with tyrosine, cysteine, histidine and lysine. In the latter three cases, there was a strong conviction of possible adduct formation.

The control samples were prepared as follows: 5.0 mg of each of the amino acid was dissolved in 0.1M acetic buffer. In next step 0.85 mg of Lim-1-OOH was dissolved in 0.42 ml of acetone. Hereafter the two solutions were mixed and the sample was incubated at 37 °C for 3 h. Then the acetone was removed by nitrogen flow and the sample was analyzed using LC/MS.

9.3.1. Reagents

Caution: the Lim-1-OOH is a strong skin sensitizer and has therefore to be handled with care!

i) 0.1M acetic buffer
0.00762 g of CH_3COONH_4 was diluted in water in 10.0 ml volumetric flask.

ii) solution of Lim-1-OOH
Lim-1-OOH was obtained from the University of Gothenburg. The whole amount of the hydroperoxide was 0.0209 g (20.9 mg). It was difficult to weight this little amount exactly. The whole amount of the obtained hydroperoxide was therefore dissolved in an aliquot amount of acetone. For each control sample 0.85 mg of limonene-1-hydroperoxide in 0.42 ml of acetone was necessary. Thus, 20.9 mg of Lim-1-OOH was diluted in 10.327 ml of acetone.

iii) amino acids
The amount of the amino acids can be read out from the Tab.10.

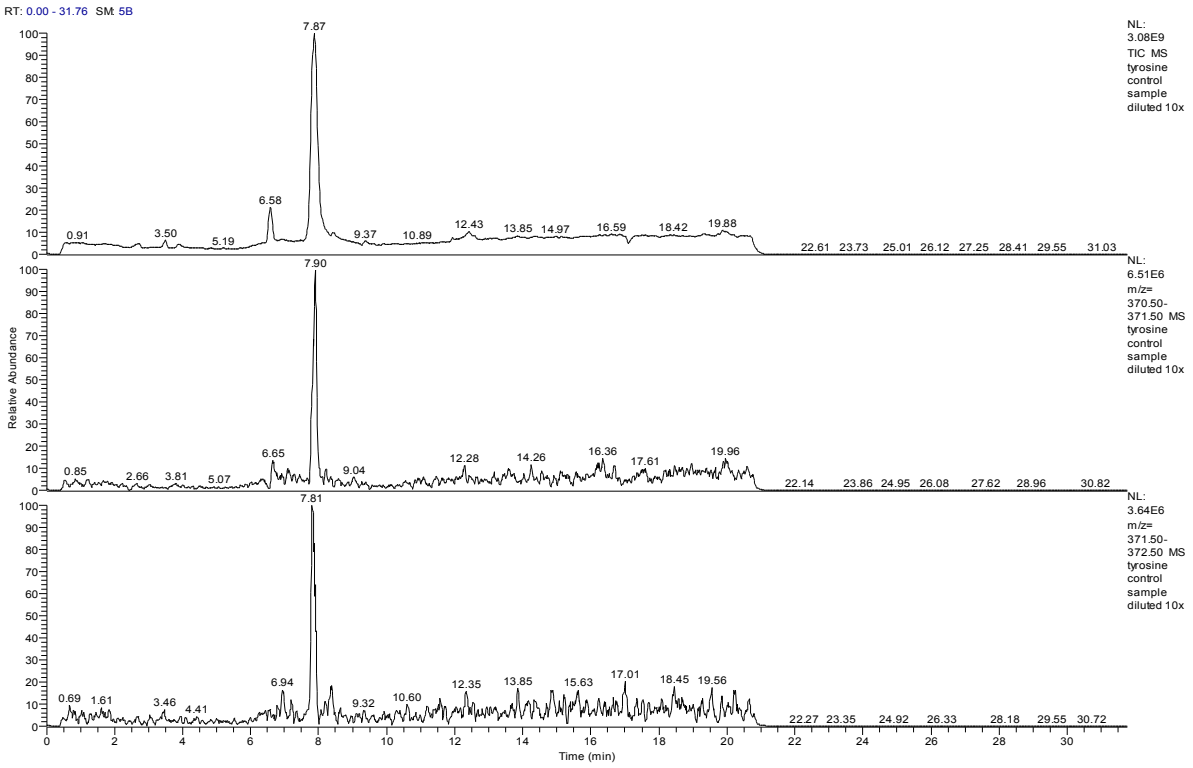
Tab.10. Amounts of the amino acids for the control samples experiments

Amino acid	*Weight*
Tyrosine	0.0052 g
Cysteine	0.0051 g
Histidine	0.0050 g
Lysine	0.0053 g

9.4. Tyrosine

Tyrosine control samples experiments were performed in order to investigate the origin of the protonated masses 371 and 372. The control sample was diluted 100x. It was already implied in the *chapter 8.6* addressed to the screening experiments of tyrosine, that both of the protonated masses were seen when the control sample was analyzed. The analysis was performed on LCQ DECA instruments using the gradient elution. Intensity of TIC was 3.08 E9 counts.

On the top: TIC of the tyrosine control sample diluted 100x , in the middle RIC of m/z 371, at the bottom: RIC of m/z 372

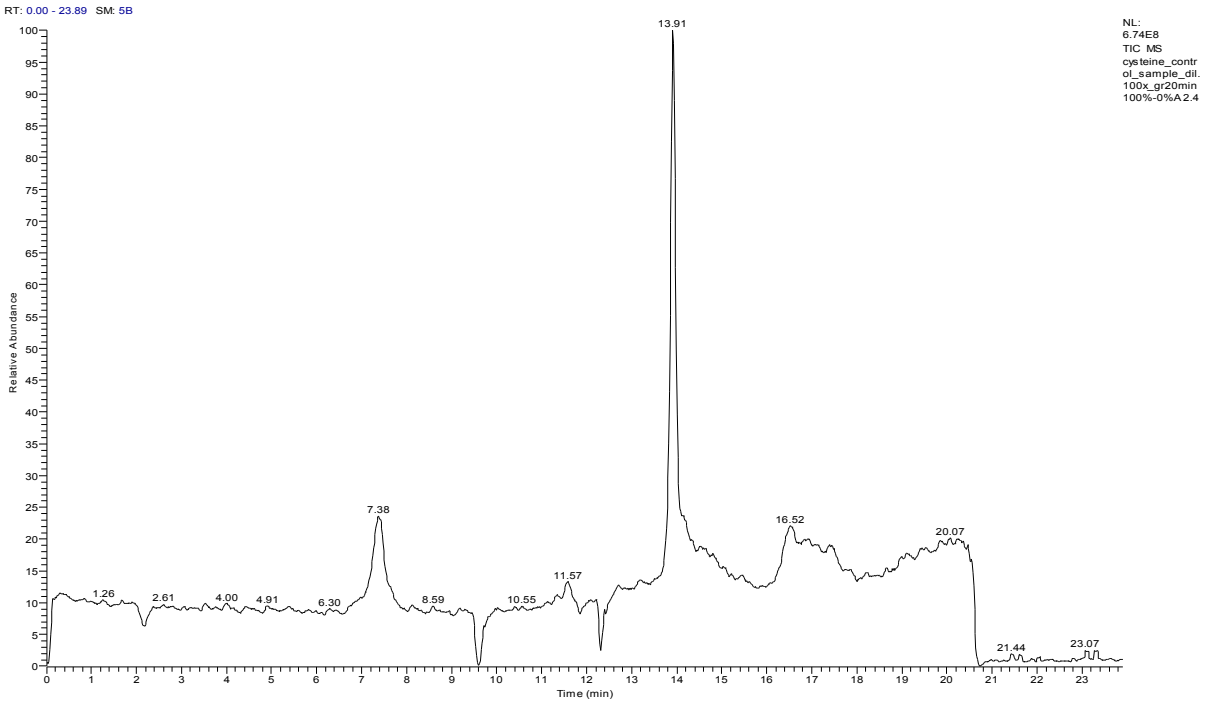


→ **Lim-1-OOH does not create adducts with tyrosine.**

9.5. Cysteine

The cysteine control sample 100x diluted was analyzed on LCQ DECA instrument. The intensity of TIC was 6.74 E8 counts. Intensities of RICs of certain m/z are summarized in Tab.11.

TIC of cysteine control sample diluted 100x



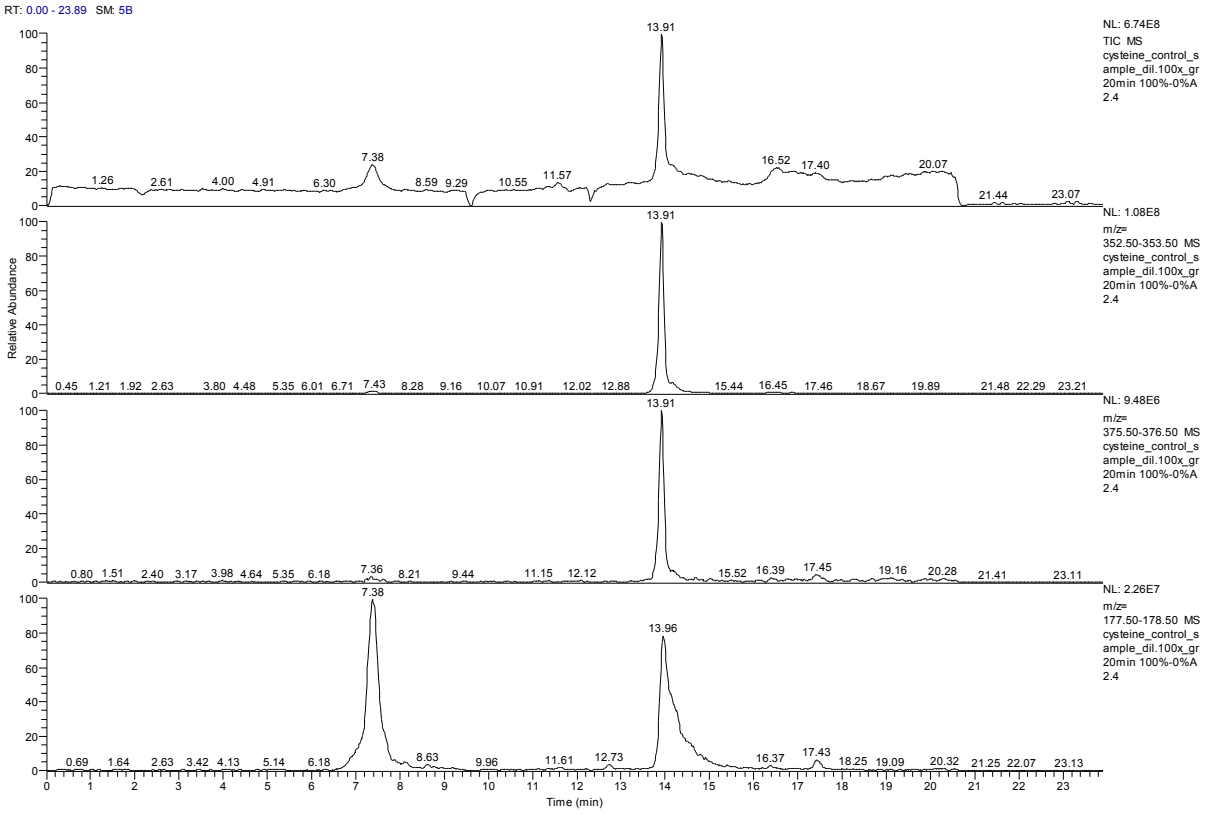
Tab.11. Intensities of RICs in cysteine control sample diluted 100x

Protonated masses	*Intensity in RIC*	*Note*
680	**1.02 E6**	**Group A, possible adduct**
636	**2.35 E5**	**Group A, possible adduct**
521	**4.40 E5**	**Group A, possible adduct**
503	**7.95 E5**	**Group A, possible adduct**
339 *	2.51 E6	Group A/C
312 *	3.08 E6	Group A/C
362	**3.11 E5**	**Group B, possible adduct**
348	**3.09 E5**	**Group B, possible adduct**
346	**7.23 E5**	**Group B, possible adduct**
330	**4.22 E5**	**Group B, possible adduct**
328	**5.21 E5**	**Group B, possible adduct**
234 *	2.22 E6	Group C
220 *	3.55 E6	Group C

N.B. * indicates straight narrow peak

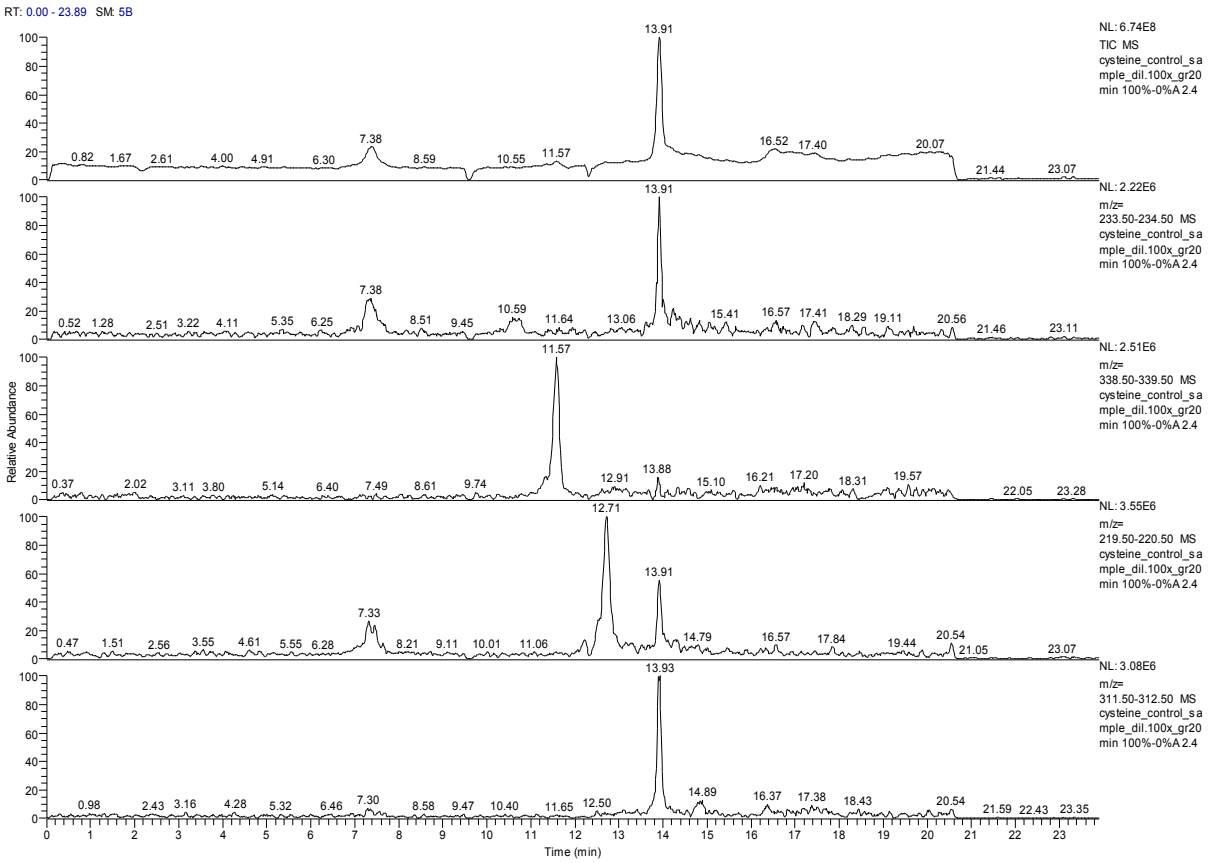
Below there are shown chromatograms of cysteine itself, of the dimer and the sodium adduct of the dimer in the control sample.

On the top: TIC of cysteine control sample diluted 100x , on the second position: RIC of m/z 353 (dimer of cys), on the third position: RIC of m/z 376 (Na^+ adduct of cys dimer), at the bottom: RIC of m/z 178 (cys).



From all the selected masses only m/z of 220, 234, 312 and 339 showed straight narrow peaks in the control sample mixture. Therefore those four protonated masses do not correspond to adducts formed via a radical pathway between Lim-1-OOH and cysteine. The other protonated masses showed no peak (results not shown.)

On the top: TIC of cysteine control sample diluted 100x , on the second position: RIC of m/z 234, on the third position: RIC of m/z 339, on the fourth position RIC of m/z 220, at the bottom: RIC of m/z 312



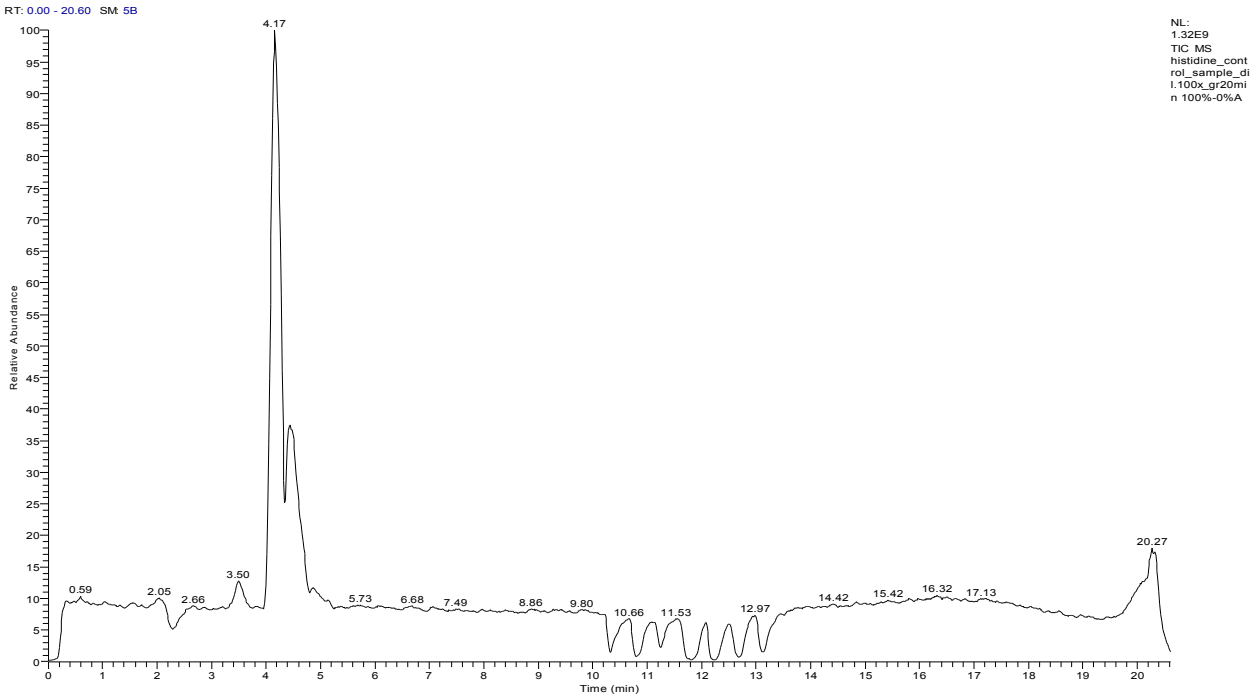
→ **Possible adducts of Lim-1-OOH with cysteine created in the presence of Fe(III) porphyrin complex correspond to the protonated masses:. 680, 636, 521, 503 and possibly also 362, 348, 346, 330 and 328. Structure of these potential adducts was examined in the third stage of the project: MS^n experiments.**

9.6. Histidine

The control sample of histidine was analyzed on LCQ DECA instrument. The gradient elution was set and the sample was diluted 100x. Intensity of TIC was 1.32 E9 counts. Intensities of the aimed protonated masses are concluded in Tab.12.

The m/z suggested as adducts or their fragments (442, 428, 426 and 410) showed no peak and were present at lower intensities (data not shown).

TIC of histidine control sample diluted 100x



Tab.12. Intensities of RICs in histidine control sample diluted 100x

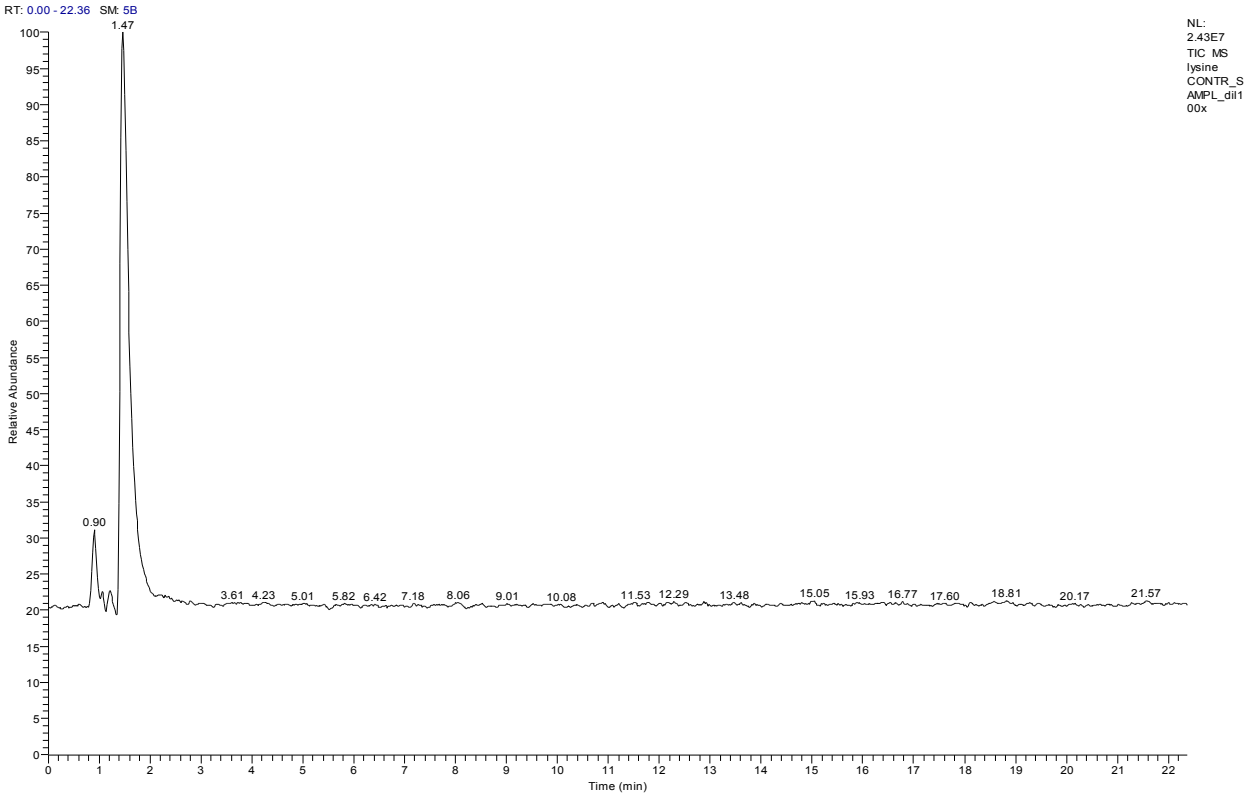
Protonated mass	*Intensity in RIC*	*Note*
442	**4.66 E5**	**Possible adduct**
428	**3.94 E5**	**Possible adduct**
426	**3.77 E5**	**Possible adduct**
410	**4.23 E5**	**Possible adduct**
260	2.49 E7	Fragment of histidine

→ **Histidine is likely to from adducts with Lim-1-OOH in the presence of the Fe (III) Porphyrin complex. The protonated masses if the aducts are: 442, 428, 426 and 410. The structure of the revealed adducts was examined in the MS^n experiments during the next stage of the project.**

9.7. Lysine

The control sample experiment of lysine 100x diluted was performed on TSQ Vantage instrument. The TIC was 2.43 E7 counts. The intensities of the focused protonated masses are summarized in Tab.13.

On the top: TIC of lysine control sample diluted 100x

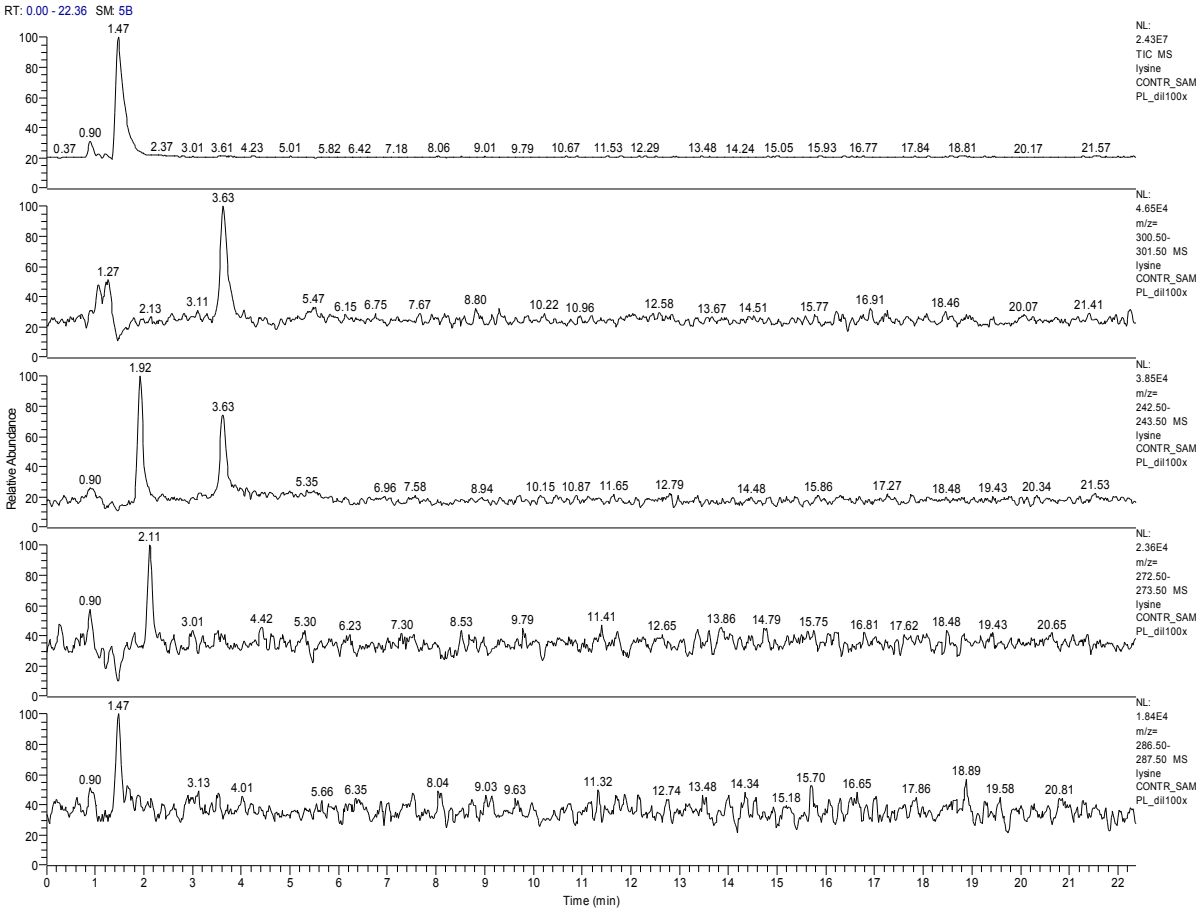


Tab.13. Intensities of RICs in lysine control sample diluted 100x

Protonated mass	*Intensity in RIC*	*Note*
444	**5.09 E3**	**Possible adduct**
411	**6.64 E3**	**Possible adduct**
371	**1.95 E4**	**Possible adduct**
321	**1.36 E4**	**Possible adduct**
353	**7.17 E4**	**Possible adduct**
339	**1.18 E4**	**Possible adduct**
311	**1.44 E4**	**Possible adduct**
301 *	4.65 E4	
287 *	1.84 E4	
273 *	2.36 E4	
243 *	3.85 E4	

N.B. * indicates straight narrow peak

In the top: TIC of lysine control sample diluted 100x, on the second position: RIC of m/z 301, on the third position: RIC of m/z 243, on the fourth position: RIC of m/z 273, at the bottom: RIC of m/z 287



→ **Lysine is considered to create adducts with limonene-1-hydroperoxide. The protonated masses of the adducts are possibly: 444, 411, 371, 353, 339, 321 and 311. The structures of the sorted out protonated masses were analyzed through MS^n experiments.**

10. MS^n experiments

10.1. Overview of the preliminary results II

The last of the three steps of the project included the determination or at least suggestion of the proposed adducts. The structure was mainly revealed during the experiments on LCQ DECA instrument, as the ion trap mass analyzer enabled tandem mass spectrometry in more stages.

After the previous steps were performed, three of the used amino acids were concluded to form adducts with Lim-1-OOH in the presence of Fe (III) Porphyrin complex. They were cysteine, histidine and lysine. Overview of the protonated masses, which might correspond to the adducts are summarized in Tab.14. The following MS^n experiments were focused on those.

Tab.14. M/z of estimated adducts of cysteine, histidine and lysine

Amino acid	*Protonated masses of the might-be adducts*
Cysteine	**680, 636, 521, 503** **362, 348, 346, 330, 328**
Histidine	**442, 428, 426, 410**
Lysine	**444, 411, 371, 353, 339, 321, 311**

Note: All the suggested structures are identified only tentatively, as true identification of the structures will need also NMR verification!

10.2. Cysteine

- Cysteine and dimer

MS^2 of 177: 76.11, 92.98, 117.97, 136.10, 150.31, 160.39

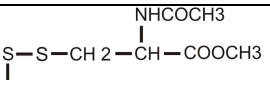
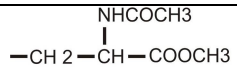
MS^2 of 353: 176.98, 209.85, 233.65, 292.89, 320.87

- Adduct of m/z 680

The fragments were obtained on LCQ DECA.

MS^2 : 471.93, 353.89, 502.96, 535.89, 662.92

Tab. 15. Fragments of cys 680

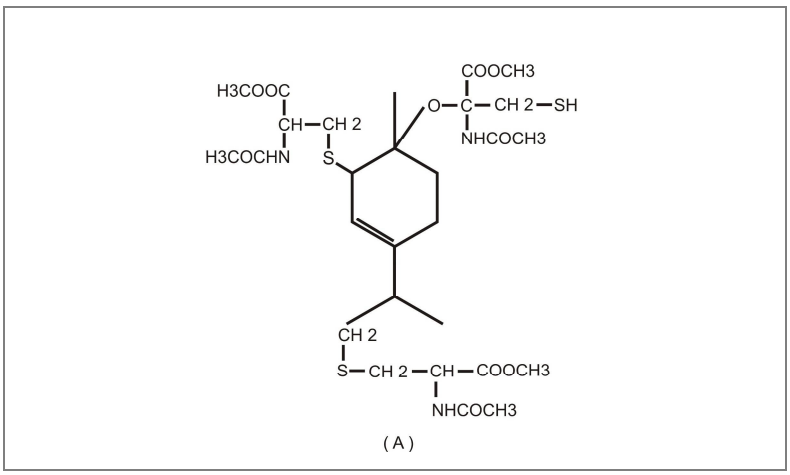
Fragment	*Loss*	*Identification*
471.93	(-208)	loss of 
353.89	(-326)	dimer of cys
502.96	(-177)	Lim-1-OOH + two molecules of cys
535.89	(-144)	loss of 
662.92	(-18)	loss of water

Tentative identification: A compound with mass 679 would correspond to one molecule of Lim-1-OOH and 3 molecules of cysteine. There are several possibilities, how to suggest such a structure.

Option A) represents one molecule of cysteine bound through the double bond of the isopropenyl group of Lim-1-OOH via thiol-ene reaction, the second molecule of cysteine bound through the double bond of the six-membered ring also via thiol-ene reaction and the third molecule of cysteine bound through the alpha carbon of the cysteine to the peroxyl group of Lim-1-OOH.

Nevertheless, in such a molecule the double bond of the six-membered ring would have to be moved to a new position in order to obtain the correct mass instead of being removed completely. (151 + 176 + 176 + 176 = 679.) Such an adduct is depicted in Fig.11. However, even though such a structure is not impossible, it is less probable.

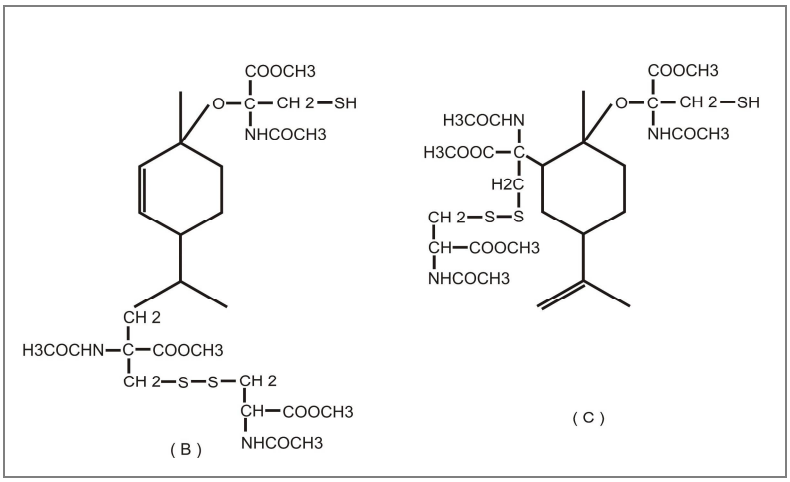
Fig.11. A possible structure of cys 679 Da showing m/z 680 (A)



The other structures would correspond to one molecule of cysteine and one molecule of cysteine dimer being bound to different parts of Lim-1-OOH. (176 + 175 + 176 + 152 = 679.) They are also more probable considering the results of MS^2, when the fragment corresponding to the cysteine dimer (353) was observed.

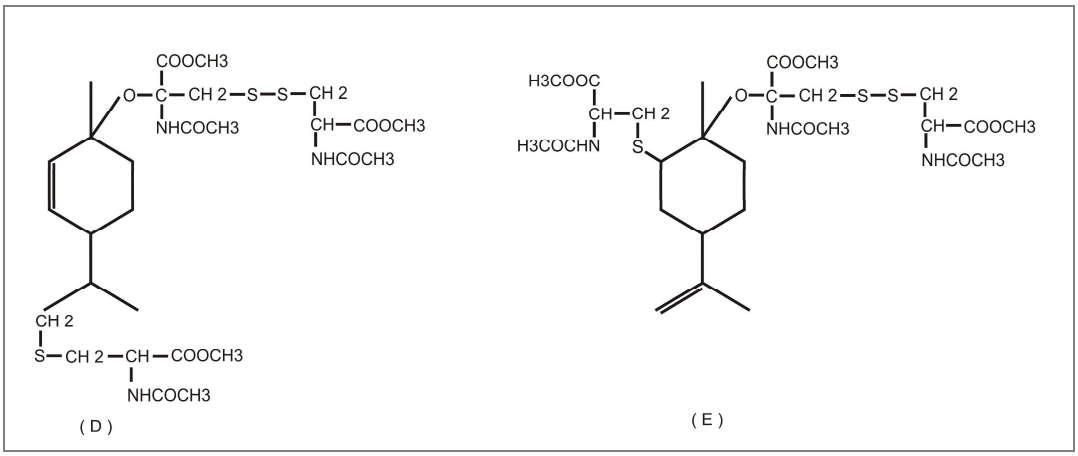
First couple is depicted in Fig.12. These structures would correspond to the molecule of cysteine bound through the alpha carbon to the peroxyl group of Lim-1-OOH and dimer of cysteine bound again through the alpha carbon of one of the cysteines to the double bond of the isopropenyl group (B) or the double bond of the six-membered ring respectively (C).

Fig.12. Possible structures of cys 679 Da showing m/z 680 (B), (C)



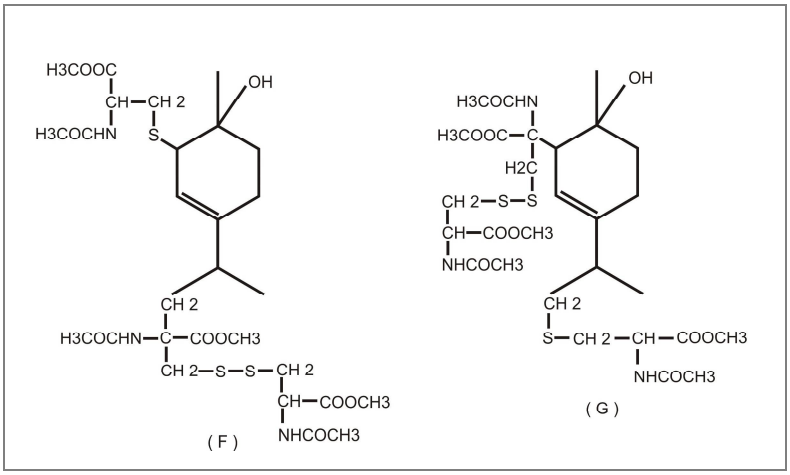
The most probable structures are shown in Fig.13. They show cysteine dimer bound through the alpha carbon to the peroxyl group of Lim-1-OOH and one molecule of cysteine bound to the double bond of the isopropenyl group (D) or to the double bond of the six-membered ring respectively (E).

Fig.13. Possible structures of cys 679 Da showing m/z 680 (D), (E)



There are also two structures shown in Fig.14., which could correspond to the m/z 680. Nevertheless, they are again less probable, as they comprise the shift of the double bond in the six-membered ring. They are represented by one molecule of cysteine bound through the double bond of the six-membered ring and dimer bound through the double bond of the isopropenyl group (F) or vice versa (G). In both cases there is a shift of the double bond in the six-membered ring. Moreover, the free peroxyl group of Lim-1-OOH is turned into hydroxyl group. (176 + 176 + 175 + 168 – 16 = 679.)

Fig.14. Possible structures of cys 679 Da showing m/z 680 (F), (G)



- Adduct of m/z 636

Fragments obtained on LCQ DECA.

MS^2: 440.04, 446.98, 492.17

MS^3 of m/z 447: 189.81 (loss of 257), 257.94, 303.93

MS^4 of m/z 304: 135.89 (loss of 168 – Lim-1-OOH?), 157.88, 178.00, 189.92, 257.73

MS^5 of m/z 190: 129.45 (loss of acetyl – 60), 147.90 (loss of 42, isopropenyl is 41) , 157.78 (loss of methanol - 32)

447 + 189 = 636

636 looses 189, 447 looses 189, 304 looses 189 – the same pattern is repeated

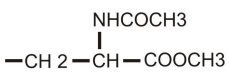
Tentative identification: The adduct corresponds to the loss of 44 from the adduct of m/z 680, but it has not been identified more exactly.

- Adduct of m/z 521

Fragments obtained on TSQ Vantage.

MS^2: 151.06, 177.06, 346.15, 287.90, 378.06, 399.07, 480.02, 488.96

Tab.16. Fragments of cys 521

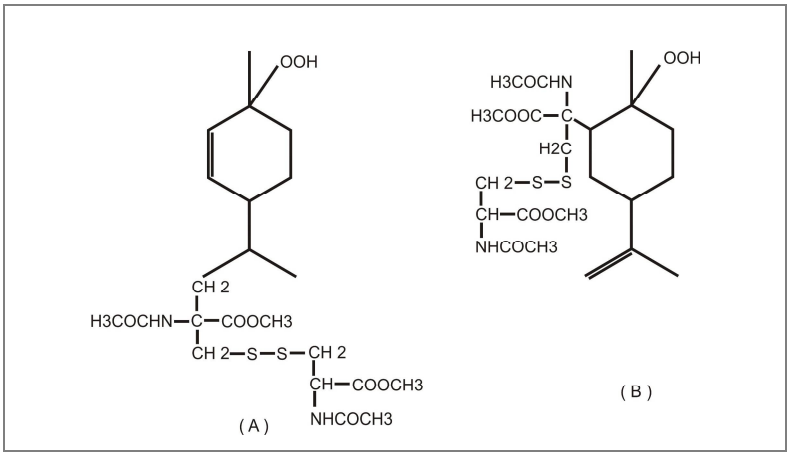
Fragment	*Loss*	*Identification*
151.06	(-370)	terpenoid moiety?
177.06	(-344)	cysteine?
346.15	(-175)	
287.90	(-233)	
378.06	(-143)	might be loss of 
399.07	(-122)	
480.02	(-41)	loss of isopropenyl
488.96	(-32)	loss of methanol

Tentative identification: The 521 protonated mass would correspond to the 503 plus a molecule of water (18) but MS^2 of 521 gave no 503.

Several clarifications of the structure are offered. However, when talking about the probability, there are some doubts. In all three cases depicted below, the structures contain one molecule of Lim-1-OOH and two molecules of cysteine.

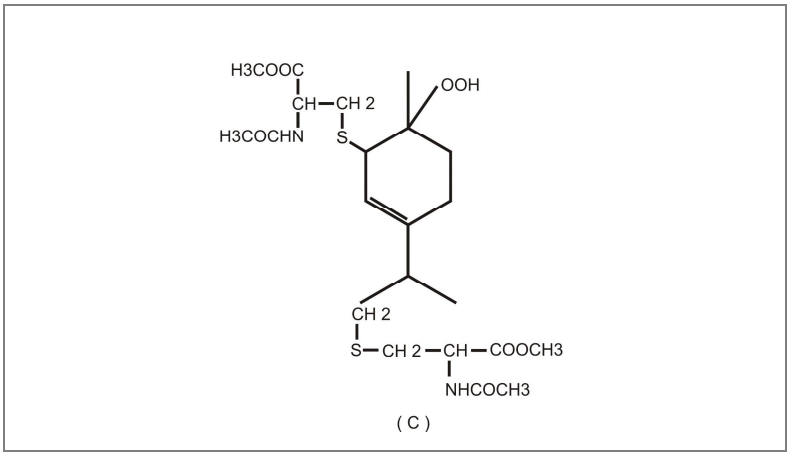
In Fig.15. there are shown two possible structures, which are represented by dimer of cysteine bound through the alpha carbon of one of the cysteine molecule to the double bond of the isopropenyl group (A) or to the double bond of the six-membered ring respectively (B). The option (B) might be supported by the loss of isopropenyl in MS^2 experiments. (176 + 175 + 169 = 520.)

Fig.15. Possible structures of cys 520 Da showing m/z 521 (A), (B)



The third option would again comprise the shift of the double bond in the six-membered ring (C). In such case, one molecule of cysteine would be bound through the double bond of the isopropenyl group and the second molecule of cysteine through the double bond of the six-membered ring. (176 + 176 + 168 = 520.) This structure is seen in Fig.16.

Fig.16. A possible structure of cys 520 Da showing m/z 521 (C)

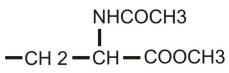


- Adduct of m/z 503

Fragments obtained on LCQ DECA. MS^2 of 680 gave the 503 fragment. In following MS^3 experiments these fragments were observed:

MS^3 of m/z 503: 164.96, 352.17, 359.72, 451.89

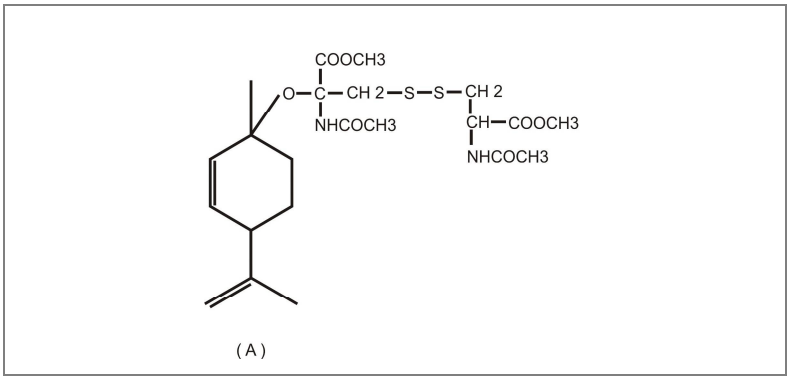
Tab.17. Fragments of cys 503

Fragment	*Loss*	*Identification*
164.96	(-338)	
352.17	(-151)	dimer of cys
359.72	(-143)	might be loss of 
451.89	(-51)	

Tentative identification: The protonated mass 503 would correspond to two molecules of cysteine bound to Lim-1-OOH. Six options are offered.

The most probable structure (A) is shown in Fig.17. Dimer of cysteine is bound through the alpha carbon of one of the cysteine molecule to the peroxyl group of Lim-1-OOH. (176 + 175 + 151 = 502). The protonated mass 503 was created also during the fragmentation of m/z 680. This fact supports the structures of 680 (E) and (D) and structure of 503 (A) depicted below, where cysteine dimer is bound to the peroxyl group of Lim-1-OOH.

Fig.17. A possible structure of cys 502 Da showing m/z 503 (A)

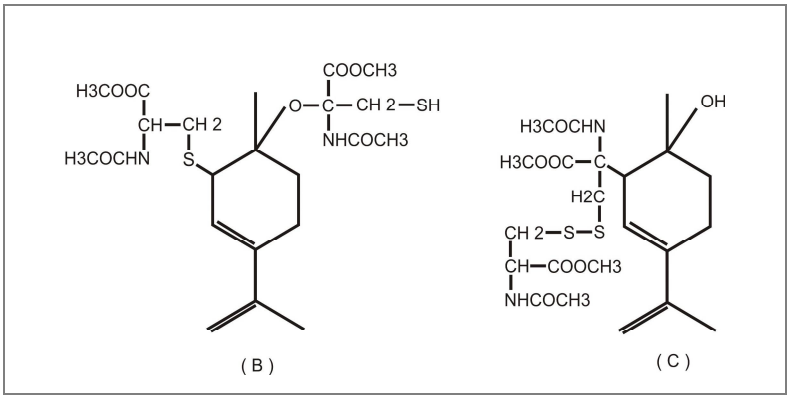


Next two, less probable though, sructures are summarized in Fig.18. (B) is represented by one molecule of cysteine bound to the peroxyl group of Lim-1-OOH through the alpha carbon and second molecule of cysteine bound to the double bond of the six-membered ring according to the thiol-ene reaction. This structure shows the shift of the double bond of the six-membered ring. (176 + 176 + 150 = 502.)

Another structural option (C) is represented by dimer bound to the double bond of the six-membered ring. The shift of the double bond in the ring is also present. Moreover the free

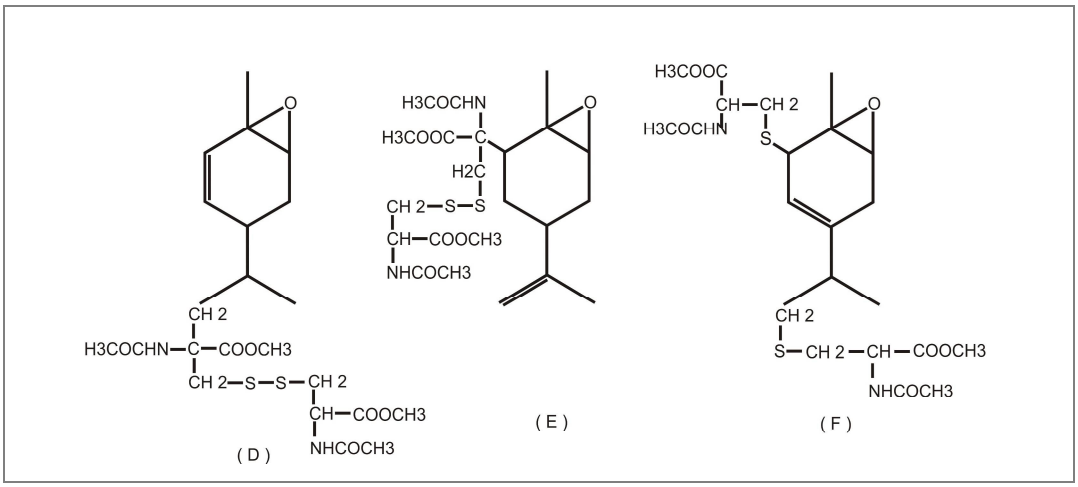
peroxyl group of Lim-1-OOH is converted into hydroxyl group. (176 + 175 + 167 – 16 = 502.)

Fig.18. Possible structures of cys 502 Da showing m/z 503 (B), (C)



In Fig.19, there are seen three structural options of m/z 503. They are derived from the m/z 521 options by replacing the peroxyl group of the Lim-1-OOH by epoxide. (176 + 175 + 169 – 18 = 502.)

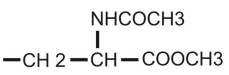
Fig.19. Possible structures of cys 502 Da showing m/z 503 (D), (E), (F)



- Adduct of m/z 362

On TSQ Vantage 362 gave several peaks of following retention times: 1.74, 2.53, 3.61, 4.41 and the highest 7.54 min. The fragments corresponding to the particular peaks are summarized in Tab.15.

Tab.18. Fragments of cys 362

RT 1.74	*RT 2.53*	*RT 3.61*	*RT 4.41*	*RT 7.54*	*Loss*	*Identification*
167.77	149.12	150.00	152.97	166.94		might correspond to terpenoid moiety
	176.25	178.17	175.99			cysteine
261.87	262.11				(-100)	
			218.99	218.95	(-143)	might be loss of 
279.91	279.97				(-82)	
297.95	299.77	300.11			(-62)	
320.93	321.11	320.70		320.07	(-41)	loss isopropenyl group
	344.16	344.11			(-18)	loss of water

Tentative identification: The 362 adduct of cysteine would stand for Lim-1-OOH with one molecule of cysteine and one additional oxygen. (176 + 169 + 16 = 361.) It has not been identified more exactly though.

- Adduct of m/z 348

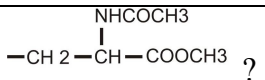
Fragments obtained on LCQ DECA

MS^2: 144.05, 152.16, 177.82, 202.88, 221.93, 239.86, 329.88

MS^3 of m/z 240: 222.02

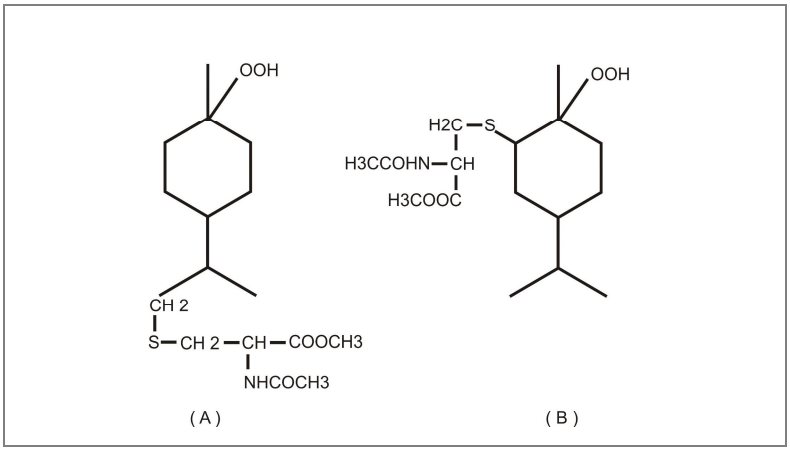
MS4 of m/z 222: 153.93, 180.93

Tab.19. Fragments of cys 348

<table>
<tr><td>Fragment MS [2]</td><td>Loss</td><td>Identification</td></tr>
<tr><td>144.05</td><td>(-204)</td><td>  ?</td></tr>
<tr><td>152.16</td><td>(-196)</td><td>terpenic moiety?</td></tr>
<tr><td>177.82</td><td>(-170)</td><td>cysteine</td></tr>
<tr><td>202.88</td><td>(-145)</td><td></td></tr>
<tr><td>221.93</td><td>(-126)</td><td></td></tr>
<tr><td>239.86
<table><tr><td>Fragment MS [3]</td><td>Loss / Ident.</td></tr><tr><td>222.02
<table><tr><td>Fragment MS [4]</td><td>Loss / Ident.</td></tr><tr><td>153.93</td><td>(-68)</td></tr><tr><td>180.93</td><td>(- 41) / loss of isopropenyl?</td></tr></table></td><td>(-18) / loss of water</td></tr></table></td><td>(-108)</td><td></td></tr>
<tr><td>329.88</td><td>(-18)</td><td>loss of water</td></tr>
</table>

Tentative identification: M/z 348 would correspond to one molecule of cysteine with one molecule of Lim-1-OOH plus two additional hydrogens, i.e. without one double bond. (176 + 169 + 2 = 347.) There are two possibilities, how to depict such a structure. Fig.20. shows both of the options: (A) cysteine bound to the double bond of the isopropenyl group, meanwhile the double bond in the six-membered ring is missing; (B) cysteine bound through the double bond of the six-membered ring and the double bond of the isopropenyl group is missing.

Fig.20. Possible structures of cys 347 Da showing m/z 348 (A), (B)



- Adduct of m/z 346

Fragments obtained on LCQ DECA.

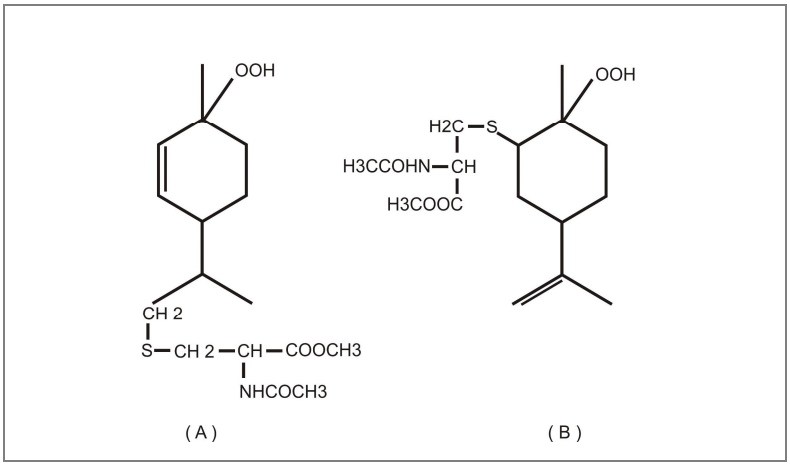
MS^2: 144.02, 152.01, 177.95, 203.09, 321.63, 328.29

Tab.20. Fragments of cys 346

Fragment	*Loss*	*Identification*
144.02	(-202)	NHCOCH3 —CH 2—CH—COOCH3 ?
152.01	(-194)	terpenic moeity?
177.95	(-168)	cysteine
203.09	(-143)	might be loss of NHCOCH3 —CH 2—CH—COOCH3
321.63	(-24)	
328.29	(-18)	loss of water

Tentative identification: M/z 346 corresponds to one molecule of cysteine bound to Lim-1-OOH with the peroxyl group preserved. (176 + 169 = 345.) There are again two options, both shown in Fig.21. (A) shows cysteine bound through the double bond of the isopropenyl group, (B) cysteine bound through the double bond of the six-membered ring of Lim-1-OOH.

Fig.21. Possible structures of cys 345 Da showing m/z 346 (A), (B)



- Adduct of m/z 330

Fragments obtained on LCQ DECA.

MS^2: 144.02, 152.94, 177.90, 270.07, 288.08, 298.01, 312.01

Fragments obtained on TSQ Vantage.

MS^2: 144.01, 153.05, 178.00, 247.85, 288.02, 298.08

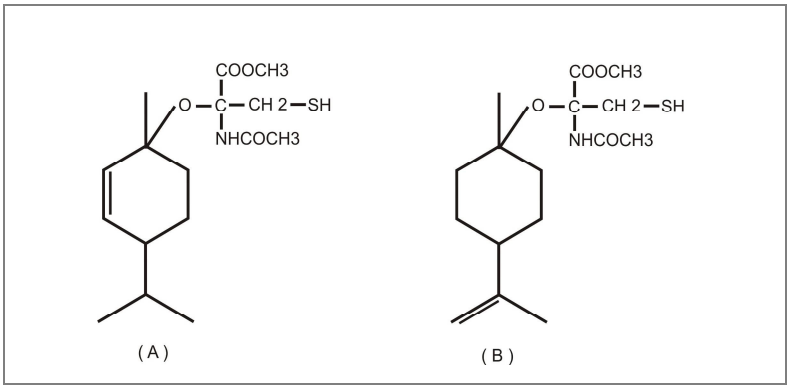
Tab.21. Fragments of cys 330

Fragment	*Loss*	*Identification*
144.02	(-186)	NHCOCH3 —CH 2—CH—COOCH3 ?
152.94	(-177)	terpenoid moeity?
177.90	(-152)	cysteine
270.07	(-60)	loss of acetyl
288.08	(-42)	might be loss of isopropenyl?
298.01	(-32)	loss of methanol
312.01	(-18)	loss of water

Tentative identification: The protonated mass 330 corresponds to the loss of water from m/z 348. There are six options of adduct with m/z 330.

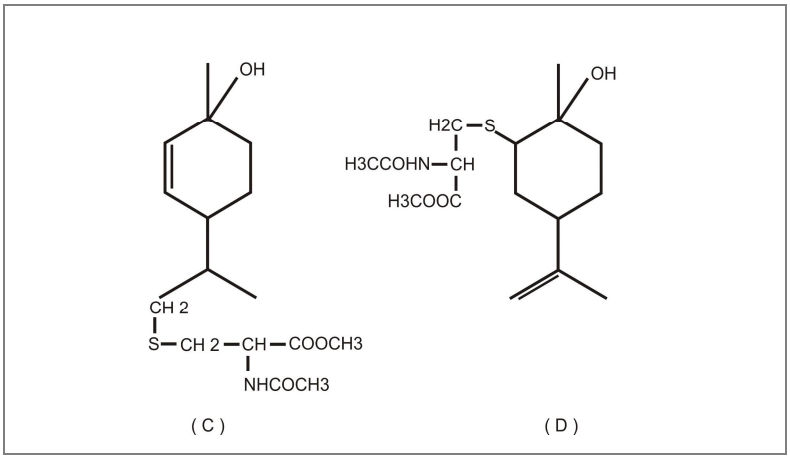
Two of the possible structures are seen in Fig.22. They comprise one molecule of cysteine bound through the alpha carbon to the peroxyl group of Lim-1-OOH. To get the correct mass, one double bond has to be missing. (176 + 151 + 2 = 329.) In option (A) it is the double bond in the isopropenyl group, the option (B) shows the double bond of the six-membered ring missing.

Fig.22. Possible structures of cys 329 Da showing m/z 330 (A), (B)



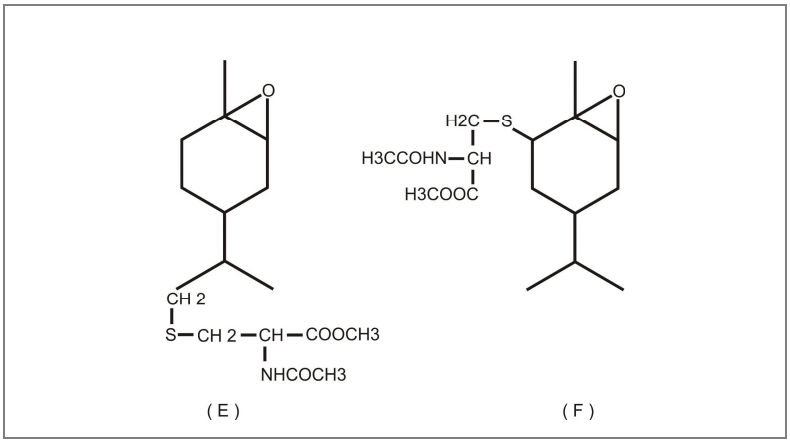
In Fig.23. there are depicted other two possible structures. They are represented by the structures seen in Fig.21. but this time the peroxyl group of Lim-1-OOH is turned into hydroxyl group. (176 + 169 – 16 = 329.) (C) represents cysteine bound trough the isopropenyl group, with the peroxyl group converted into hydroxyl group; (D) cysteine bound through the double bond of the six-membered ring, with the peroxyl group converted into hydroxyl group.

Fig.23. Possible structures of cys 329 Da showing m/z 330 (C), (D)



Finally Fig.24. shows the last two options. They are represented by structures depicted in Fig.20. but this time the peroxyl group of Lim-1-OOH is converted into epoxide. One molecule of cysteine is bound to the double bond of either the isopropenyl group (E), or of the six-membered ring (F) meanwhile one particular double bond is missing. (176 + 169 + 2 – 18 = 329.)

Fig.24. Possible structures of cys 329 Da showing m/z 330 (E), (F)



- Adduct of m/z 328

Fragments obtained on LCQ DECA.

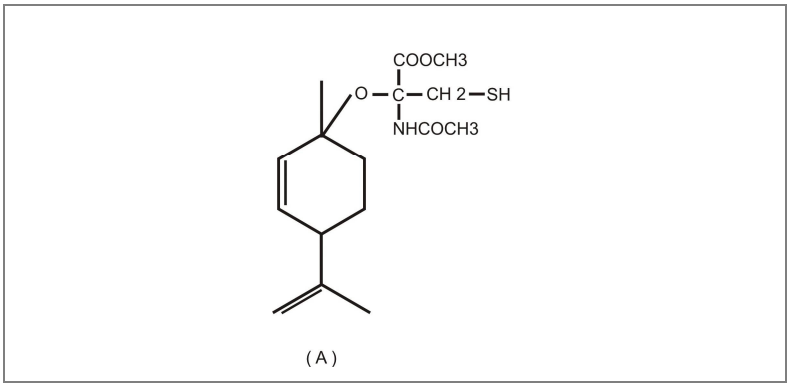
MS^2: 135.10, 144.03, 151.19, 177.93, 234.05, 286.67

Tab.22. Fragments of cys 328

Fragment	*Loss*	*Identification*
135.10	(-193)	fragment of cysteine
144.03	(-184)	NHCOCH3 —CH 2—CH—COOCH3 ?
151.19	(-177)	terpenoid moeity?
177.93	(-150)	cysteine
234.05	(-94)	
286.67	(-41)	loss of isopropenyl

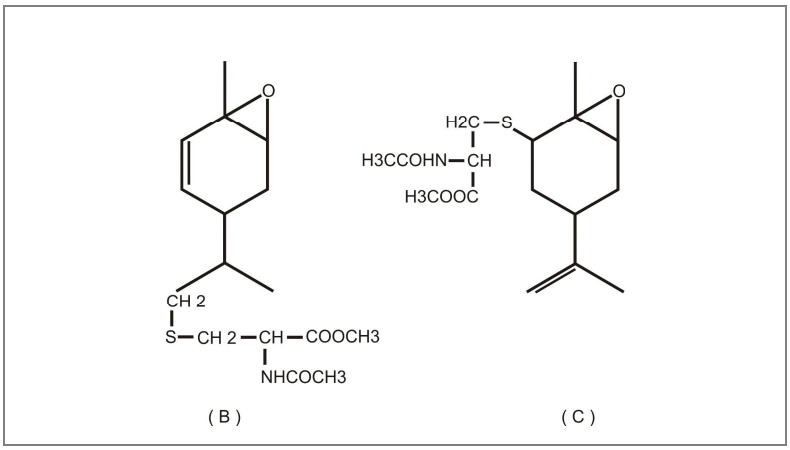
Tentative identification: The adduct of m/z 328 offers three structural possibilites. The first one is depicted in Fig.25. The molecule of cysteine is bound through the alpha carbon to the peroxyl group of Lim-1-OOH, both double bond are preserved. (176 + 151 = 327.)

Fig.25. A possible structure of cys 327 Da showing m/z 328 (A)



In Fig.26. there are shown the other two tentative explanations of m/z 328. They are represented by the structures seen in Fig.21. but this time the peroxyl group is turned into epoxide. Meanwhile one molecule of cysteine is bound to the double bond either of the isopropenyl group (B) or of the six-membered ring (C). (176 + 169 – 18 = 327.)

Fig.26. Possible structures of cys 327 Da showing m/z 328(B), (C)



10.3. Histidine

- Adduct of m/z 442

The fragments were obtained on LCQ DECA.

MS^2: 152.76, 167.22, 196.11, 214.00, 274.07, 303.16, 364.19, 382.20, 410.10, 424.21

MS^3 of m/z 424: 166.97, 245.13, 303.12, 321.06, 348.17, 364.13, 392.07

Tab.23. Fragments of his 442

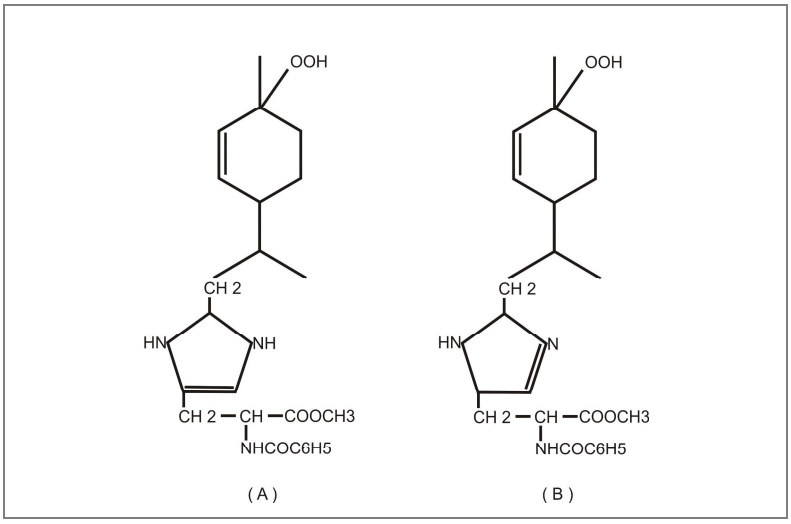
Fragment MS [2]	*Loss*	*Identification*
152.76	(-289)	
167.22	(-275)	
196.11	(-246)	
214.00	(-228)	fragment of histidine – without acetyl
274.07	(-168)	histidine
303.16	(-139)	
364.19	(-78)	loss of phenyl C_6H_5
382.20	(-60)	loss of acetyl
410.10	(-32)	loss of methanol
424.21	(-18)	loss of water

Fragment MS [3]	*Loss / Ident.*
166.97	(-257)
245.13	(-179)
303.12	(-121) / loss of protected amino group
321.06	(-103)
348.17	(-76)
364.13	(-60)
392.07	(-32)

Tentative identification: Adduct of m/z 442 would correspond to one molecule of histidine and one molecule of Lim-1-OOH (273 + 168 = 441.) The peroxyl group of Lim-1-OOH would have to be preserved.

Histidine would seem to be bound to Lim-1-OOH in several possible ways. However, there is only one, which is the most probable. Histidine is the most likely bound to the hydroperoxide through one of the imidazole carbons. In Fig.27. therefore is depicted the most probable option of histidine adduct of m/z 442.

Fig.27. Possible structures of his 441 Da showing m/z 442 (A), (B)



- Adduct of m/z 428

The fragments were obtained on LCQ DECA.

MS^2: 196.14, 213.91, 274.14, 307.15, 350.21, 368.12, 410.15, 426.13

MS^3 of m/z 410: 213.93, 260.11, 273.98, 288.87, 334.03, 350.14, 378.11, 391.98

Tandem MS was also performed on TSQ Vantage.

MS^2: 196.05, 214.04, 260.08, 278.10, 350.12, 368.19, 382.25, 410.20

Tab.24. Fragments of his 428

<table>
<tr><th>Fragment MS ²</th><th>Loss</th><th>Identification</th></tr>
<tr><td>196.14</td><td>(-232)</td><td></td></tr>
<tr><td>213.91</td><td>(-214)</td><td>fragment of histidine – without acetyl</td></tr>
<tr><td>274.14</td><td>(-154)</td><td>histidine</td></tr>
<tr><td>307.15</td><td>(-121)</td><td>loss of protected amino group $NHCOC_6H_5$</td></tr>
<tr><td>350.21</td><td>(-78)</td><td>loss of phenyl C_6H_5</td></tr>
<tr><td>368.12</td><td>(-60)</td><td>loss of acetyl</td></tr>
<tr><td>410.15
<table>
<tr><th>Fragment MS ³</th><th>Loss / Ident.</th></tr>
<tr><td>213.93</td><td>(-196)</td></tr>
<tr><td>260.11</td><td>(-150)</td></tr>
<tr><td>273.98</td><td>(-154) / histidine</td></tr>
<tr><td>288.87</td><td>(-121) / loss of protected amino group</td></tr>
<tr><td>334.03</td><td>(-76)</td></tr>
<tr><td>350.14</td><td>(-60) / loss of acetyl</td></tr>
<tr><td>378.11</td><td>(-32) / loss of methanol</td></tr>
<tr><td>391.98</td><td>(-18) / loss of water</td></tr>
</table></td><td>(-18)</td><td>loss of water</td></tr>
<tr><td>426.13</td><td>(-2)</td><td></td></tr>
</table>

Tentative identification: The structural options of m/z 428 might be derived from the m/z 426, which is discussed later. It would comprise the options with one double bond missing, i.e. with two additional hydrogens. Nevertheless, after MS^n experiments, it is quite probable, that m/z of 428 does not represent any adduct of histidine and Lim-1-OOH. It is rather a cluster of two histidine fragments of m/z 214. However, it is disputable, why such a structure was not seen also in the control sample experiment.

- Adduct of m/z 426

The fragments were obtained on LCQ DECA.

MS^2: 214.00, 241.85, 260.03, 274.06, 305.10, 338.07, 348.32, 366.09, 394.09, 408.07

Tandem MS was also performed on TSQ Vantage.

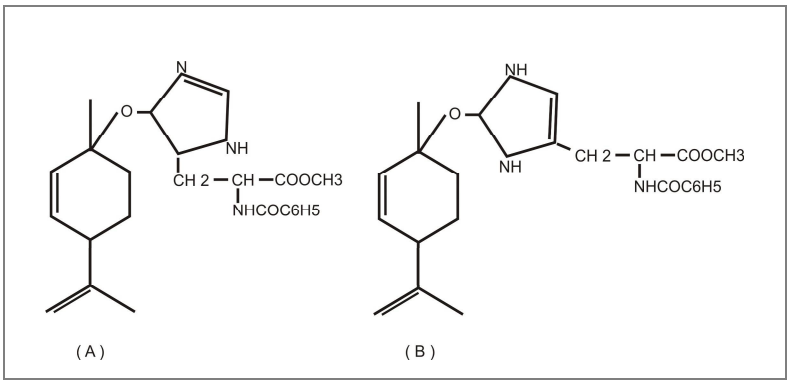
MS^2: 153.04, 166.97, 196.06, 214.05, 241.96, 259.98, 274.03, 337.27, 347.94, 366.20, 380.07, 394.19, 408.35

Tab.25. Fragments of his 426

Fragment MS	*Loss*	*Identification*
153.04	(-273)	terpenic moiety?
166.97	(-259)	loss of his fragment
196.06	(-230)	
214.05	(-212)	fragment of histidine – without acetyl
241.96	(-184)	
259.98	(-166)	fragment of histidine
274.03	(-152)	histidine
337.27	(-89)	
347.94	(-78)	loss of phenyl C_6H_5
366.20	(-60)	loss of acetyl
380.07	(-46)	
394.19	(-32)	loss of methanol
408.35	(-18)	loss of water

Tentative identification: Although there is quite a logical explanation of such a structure offered: two histidine fragments of m/z 214 joined with a bond, the protonated mass 426 stands very probably for adduct between histidine and Lim-1-OOH. The fragment obtained during MS^2 experiments of m/z 152 might correspond to a terpenoid moiety. Adduct of such m/z may look like one of the below suggested options. Fig.28. shows the structure, as it was suggested by the team form the University of Gothenburg.

Fig.28. Possible structures of his 425 Da showing m/z 426 (A), (B)



- Adduct of m/z 410

The fragments were obtained on LCQ DECA. M/z 410 was obtained in MS^2 experiments with m/z 428. During MS^3 experiments, following fragments were observed:
MS^3 of m/z 410: 213.93, 260.11, 273.98, 288.87, 334.03, 350.14, 378.11, 391.98

Tab.26. Fragments of his 410

Fragment MS^3	*Loss*	*Identification*
213.93	(-196)	
260.11	(-150)	
273.98	(-154)	histidine
288.87	(-121)	loss of protected amino group $NHCOC_6H_5$
334.03	(-76)	
350.14	(-60)	loss of acetyl
378.11	(-32)	loss of methanol
391.98	(-18)	loss of water

Tentative identification: The m/z 410 very probable does not stand for adduct between Lim-1-OOH and histidine. It is rather a fragment from m/z 428, which was suggested to be a cluster of two histidine fragments.

10.4. Lysine

- Lysine

MS^2 of 203: 129.04, 143.98, 152.98, 161.03, 171.01, 185.98

- Adduct of m/z 444

The fragments were obtained on TSQ Vantage.
MS^2: 142.03, 184.06, 203.08, 386.20

Tentative identification: According to the fragments obtained during the MS^n experiments, the protonated mass 444 is rather considered to be an oligomer of lysine.

- Adduct of m/z 411

The fragments were obtained on TSQ Vantage.
MS^2: 110.91, 169.94, 202.90, 266.93, 295.04, 335.42, 353.12, 393.15

Tab.27. Fragments of lys 411

Fragment MS	*Loss*	*Identification*
110.91	(-300)	loss of lysine fragment / oligomer
169.94	(-241)	
202.90	(-208)	lysine
266.93	(-144)	loss of lysine fragment
295.04	(-116)	
335.42	(-76)	
353.12	(-58)	might be loss of $NHCOCH_3$ (*see Adduct of m/z 353)*
393.15	(-18)	loss of water

Tentative identification: The fragments show, that even m/z of 411 would rather correspond to some kind of lysine oligomer. Moreover another considered fragment is derived from m/z 411 (m/z of 353).

- Adduct of m/z 371

The fragments were obtained on TSQ Vantage.
MS^2: 129.05, 144.08, 154.08, 171.07, 186.09, 203.07, 279.11, 323.18, 335.18, 353.19 (loss of water), the underlined fragments correspond to the fragments of lysine

Tentative identification: Although such adduct might correspond to one molecule of lysine bound to one molecule of Lim-1-OOH (168 + 202 = 370), it is again rather an oligomer of lysine, as no terpenoid moiety is not present among the fragments.

- Adduct of m/z 339

The fragments were obtained on TSQ Vantage.
MS^2: 143.96, 162.62, 180.62, 203.35, 281.17 (loss of $NHCOCH_3$), 321.19 (loss of water), the underlined fragments correspond to the fragments of lysine

Tentative identification: M/z of 339 is considered to be another oligomer of lysine. Moreover it gives fragment of 321, which had been also suggested as adduct.

- Adduct of m/z 353

The fragments were obtained on TSQ Vantage.
MS^2: 134.97, 175.95, 177.97, 219.68, 224.88, 251.00, 270.96, 293.14 (loss of acetyl), 311.01, 334.98 (loss of water)

Tentative identification: This m/z is rather considered to be a fragment of the previous protonated masses. The protonated mass 353 was observed when MS^2 of 371 and 411 was performed. The 353 protonated mass corresponds to the loss of water (-18) from 371 and to the loss of the protected amino group of lysine ($NHCOCH_3$) (-58) from 411, respectively.

- Adduct of m/z 321

The fragments were obtained on TSQ Vantage.

MS^2: 84.05, 126.05, 144.08, 154.16, 159.91, 185.96, 218.70, 302.96 (loss of water), the underlined fragments correspond to the fragments of lysine

Tentative identification: Also the protonated mass 321 is considered to be a fragment of the previous protonated masses. The m/z 321 was observed when the MS^2 of the protonated mass 339 was performed. The m/z 321 corresponds to a loss of water (18) from 339.

- Adduct of m/z 311

The fragments were obtained on TSQ Vantage.

MS^2: 84.07, 129.05, 144.08, 171.09, 186.07, 203.05, 251.07 (loss of acetyl), 293.10 (loss of water), the underlined fragments correspond to the fragments of lysine

Tentative identification: The fragments also suggest the m/z of 311 being rather an oligomer of lysine. Moreover, the protonated mass 321 gave the fragment 311.

PART 3 – Conclusions

11. Results and discussion

The aim of the project was to examine the reactivity of strong skin allergen limonene-1-hydroperoxide with amino acids. In the presence of Fe (III) porphyrin complex used as a radical initiator, the hydroperoxide forms adducts with amino acids. Structure of these adducts was about to be suggested and/or determined.

The project involved three stages. In the first one called screening experiments, amino acids which could potentially create adduct with Lim-1-OOH were picked up. They appeared to be tyrosine, cysteine, histidine and lysine.

The second stage of control samples experiments confirmed potentially adduct-forming amino acids. After the control sample experiments cysteine, histidine and lysine remained aimed. The second stage revealed which particular protonated masses might correspond to adducts between Lim-1-OOH and amino acids.

In the last stage – MS^n experiments – the chosen protonated masses were examined. After the third stage, some of the protonated masses were confirmed as potential adduct and their structure was suggested. For true identification of the structures the NMR will also be included.

The adduct formation was after all seen only with two amino acids: cysteine and histidine. Meanwhile cysteine forms high amount of different adducts, histidine forms only two different adducts on lower amounts. Lysine was suspected to form adducts, nevertheless the MS^n experiments did not confirm any. Lysine rather forms different oligomers.

12. Conclusions

After performing all three stages of the project, several conclusions can be made.

i) Formation of adducts between limonene hydroperoxides and amino acids is strongly suspected to run via radical pathway.
ii) From the seven chosen amino acids, only cysteine and histidine showed adduct formation via a radical pathway.
iii) Tyrosine formed huge amounts of dimer and is prone to polymerization.
iv) Lysine formed huge amounts of different oligomers, but only in the presence of the radical initiator.

v) Cysteine forms broad number of different adducts of following m/z: 680, 636, 521, 503, 362, 348, 346, 330 and 328. Structures of these adducts were suggested using LC/ESI-MSMS, with the exception of the protonated masses 636 and 362. True identification needs NMR verification though.

vi) Histidine forms lower amount of adducts of following m/z: 442 and 426. Structures of these adducts were suggested using LC/ESI-MSMS. True identification needs NMR verification.

13. Souhrn

Cílem projektu bylo podrobně prozkoumat reaktivitu alergenu limonen-1-hydroperoxidu s aminokyselinami. Limonen-1-hydroperoxid vytváří s některými aminokyselinami adukty, jejichž vznik je podstatou kontaktní alergie. Předpokládá se, že tyto adukty vznikají pouze radikálovým mechanismem, tzn. pouze v přítomnosti radikálového induktoru. Zjistit strukturu případných aduktů byl druhý cíl této práce.

Celý projekt zahrnoval tři fáze. V první (screening experiments) byly vytipovány aminokyseliny, které by mohly vytvářet adukty s limonen-1-hydroperoxidem. Byly to tyrosin, cystein, histidin a lysin.

Ve druhé fázi (control samples experiments) se potvrdilo, které aminokyseliny skutečně mohou vytvářet adukty. Po této fázi zůstaly zaměřené pouze tři: cystein, histidin a lysin. Druhá fáze zároveň zahrnovala výběr m/z, které by mohly odpovídat aduktům vytvořeným v přítomnosti radikálového induktoru.

V poslední fázi byla určována struktura vybraných m/z. Některé m/z byly skutečně potvrzeny jako adukty a byla navržena jejich struktura. Nicméně ke skutečnému potvrzení těchto struktur bude třeba provést NMR analýzu.

Po provedení všech tří fází mohou být shrnuty následující fakta:

i) hydroperoxidy limonenu jsou schopny vytvářet adukty s aminokyselinami prostřednictvím radikálů.

ii) ze sedmi vybraných aminokyselin pouze cystein a histidin má schopnost tvorby aduktů radikálovým mechanismem

iii) tyrosin potvrdil schopnost polymerizace tvorbou relativně vysokého množství dimeru a trimeru.

iv) po první i druhé fázi se předpokládalo, že lysin vytváří různorodé adukty s limonen-1-hydroperoxidem. Nicméně po provedení MS^n experimentů se ukázalo, že lysin vytváří v přítomnosti radikálového induktoru mnoho různých oligomerů, nikoliv aduktů s terpenickým zbytkem, jenž by odpovídal limonen-1-hydroperoxidu.

v) cystein vyváří několik různých aduktů radikálovým mechanismem. Jejich m/z jsou 680, 636, 521, 503, 362, 348, 346, 330 a 328. Struktura těchto aduktů byla navržena po analýze výsledků MS^n experimentů, a to s výjimkou m/z 636 a 362, jejichž struktura zůstala zcela neznámá.

vi) histidin vytváří podstatně menší množství aduktů než cystein. Jejich m/z jsou 442 a 426. Také struktura těchto aduktů byla osvětlena během MS^n experimentů. Nicméně, jak už bylo poznamenáno na jiném místě, navržené struktury musí být potvrzeny také NMR analýzou.

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Sources of pictures

Fig.3. ESI: Taylor cone and forming of the droplets

http://upload.wikimedia.org/wikipedia/commons/3/3f/Ionisation_electrospray.png

date of the visit: January, 2011

Fig.4. ESI: Evaporation of the droplet

http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial_files/image006.jpg

date of the visit: January, 2011

Fig.5. Scheme of quadrupole mass analyzer

http://www.chm.bris.ac.uk/ms/images/quad-schematic2.gif

date of the visit: January, 2011

Appendix

I. List of figures

II. List of tables