Universidade de Lisboa

Faculdade de Farmácia

Stability of Horseradish Glucosinolates/ Isothiocyanates in Combination with Different Plant Extracts

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Mestrado Integrado em Ciências Farmacêuticas

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Trabalho de Campo de Mestrado Integrado em Ciências Farmacêuticas

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Resumo

O uso do rábano picante, *Armoracia rusticana* P. Gaertn., B. Mey. & Scherb., pertencente à família Brassicaceae, remonta ao tempo dos Gregos e Romanos. O rábano picante tornou-se popular na Europa pelos seus efeitos medicinais e como parte da dieta. Atualmente, é cultivado em todo mundo e utilizado maioritariamente em culinária devido ao sabor acre da sua raiz e folhas. Como planta medicinal, o rábano picante é ainda utilizado em vários países para o tratamento de dor de cabeça, pressão arterial elevada, bronquite, tosse e infeções urinárias.

O rábano picante é rico em glucosinolatos – glicosídeos que contêm enxofre, sendo os mais abundantes o *sinigrin* (que representa cerca de 80% do total de glucosinolatos) e o *gluconasturtiin*. Os glucosinolatos e os seus produtos de degradação são os responsáveis pelo sabor pungente do rábano, como também pelas suas propriedades medicinais. Especificamente, as propriedades medicinais descritas têm sido associadas aos produtos maioritários de degradação dos glucosinolatos conhecidos como isotiocianatos. Os isotiocianatos têm sido estudados devido à variedade das suas atividades biológicas das quais se destacam as atividades antimicrobiana e anticancerígena. Estes compostos são bastante reativos, propriedade que lhes confere as suas atividades biológicas, sendo, no entanto, instáveis em soluções aquosas.

Tendo em mente o estudo de uma nova abordagem para a estabilização dos isotiocianatos do rábano, foram misturados com rábano picante moído extratos aquosos de cinco espécies da família *Lamiaceae* - *Origanum vulgare* (orégãos), *Rosmarinus officinalis* (rosmaninho), *Salvia officinalis* (sálvia), *Saturejae montanae* (segurelha) e *Thymus vulgaris* (timo), tendo o decréscimo dos isotiocianatos sido estudado ao longo do tempo. Os diferentes extratos obtidos influenciaram de forma diferente a estabilidade dos isotiocianatos. Os extratos de orégãos e rosmaninho mostraram-se os mais ativos a estabilizar os compostos em estudo. A composição total em compostos fenólicos e a capacidade de sequestrar o radical DPPH foram avaliadas nos extratos aquosos das diferentes espécies em estudo com o objetivo de as relacionar com a sua atividade na degradação dos isotiocianatos. A composição total em compostos fenólicos foi comparável com os resultados obtidos para a estabilização dos isotiocianatos – os orégãos e o rosmaninho apresentaram a maior concentração destes compostos. Por sua vez, a capacidade de sequestrar o radical DPPH não apresentou relação direta com os resultados obtidos para a estabilização dos isotiocianatos - a sálvia e o rosmaninho apresentaram a maior atividade, indicando que este mecanismo não tem grande influência na degradação dos isotiocianatos.

Palavras Chave: Rábano Picante, *Armoracia rusticana*, Lamiaceae, isotiocianatos, antioxidantes.

Abstract

Horseradish, *Armoracia rusticana* P. Gaertn., B. Mey. & Scherb., from the Brassicaceae family has been used by humans for a long period of time, back to the ancient Greeks and Romans. Horseradish use was popular in Europe as a medicinal herb for the treatment of several diseases and as a part of the diet. Nowadays, horseradish is cultivated worldwide and is mainly used in culinary due to the harsh flavour of its root and leaves. As a medicinal herb, horseradish is still used in several countries for the treatment of headache, high blood pressure, bronchitis, cough and urinary infections.

Horseradish is rich in glucosinolates– sulphur-containing glycosides, being sinigrin (usually represents over 80% of the total glucosinolates) and gluconasturtiin the main representatives in the horseradish. Glucosinolates and their breakdown products are the responsible for the pungent taste and the medicinal properties of horseradish. In particular, the medicinal properties of horseradish have been associated to the main glucosinolates breakdown products known as isothiocyanates. Isothiocyanates have been described to have several biological activities that include antimicrobial and chemoprotective. isothiocyanates are reactive compounds that are unstable in aqueous solutions.

Studying a novel approach to the stabilization of horseradish isothiocyanates, aqueous extracts of five Lamiaceae family species – *Origanum vulgare* (oregano), *Rosmarinus officinalis* (rosemary), *Salvia officinalis* (sage), *Saturejae montanae* (satureja) e *Thymus vulgaris* (thyme) were mixed with grinded horseradish, and the isothiocyanates decrease over time was studied. It was found that the diverse plant extracts influenced in a different matter the stability of isothiocyanates. Oregano and rosemary aqueous extracts were found to be the most active on stabilizing the compounds. The total phenolic content and DPPH scavenging capacity were tested for the aqueous extracts of the different Lamiaceae members trying to relate it with the stabilization activity, and even though the total phenolic was comparable with the results obtained with those obtained for the stabilization of isothiocyanates - oregano and rosemary presented the higher phenolic content, the capacity of scavenging differed – sage and rosemary were the ones with highest activity, indicating that this mechanism does not have a great influence in isothiocyanates degradation.

Key words: Horseradish, *Armoracia rusticana*, Lamiaceae, isothiocyanates, antioxidants.

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Abreviatures

abs – Absorbance

AIF – Apoptosis Induction Factor

AITC - Allyl isothiocyanate

DNA – Deoxyribonucleic Acid

DPPH – 1,1-diphenyl- 2-picrylhydrazyl

GC – Gas Chromatography

H_2O – Water

HPLC – High performance liquid chromatography

JNK – c-Jun N-terminal kinase

MAPKs – Mitogen Activated Protein Kinases

MeOH – Methanol

MS – Mass Spectrometry

Na_2CO_3 – Sodium Carbonate

PDA - Diode Array Detectors

PEITC – Phenylethyl isothiocyanate

RNA – Ribonucleic Acid

SPE -Solid Phase Extraction

SPME – Solid Phase Microextraction

w/w – Weight/Weight

1. Introduction

1.1. *Armoracia rusticana*

Armoracia rusticana P. Gaertn., B. Mey. & Scherb., usually known as horseradish, is a plant that belongs to the Brassicaceae family, which contains more than 350 genera, with about 3000 species. Its root and leaves have been used as food condiment and for medical purposes. The exact origin of this species is unknow, but the records indicate its probable origin to be in Eastern Europe and West Asia. Nowadays horseradish has become natural worldwide (mainly Europe and North America), and it is possible to be found both as cultivated and wild growing (1,2).

Horseradish is a large-leaved, hardy and glabrous perennial herb that grows to a height of up to 120 cm, with a fleshy, pungently aromatic, fusiform or cylindrical taproot. Basal leaves are long-petioled, with elliptic-ovate or oblong-lanceolate, bluish green, cordate at the base and unevenly crenate lamina, that grows to a length of 30 to 100 cm (Figure 1). The lower cauline leaves have shorter petioles and may be lobed or comb-shaped-pinnate, they have linear-oblong, entire-margined, or serrate sections. The upper cauline leaves have narrow bases and are mostly sessile, oblong or lanceolate, unevenly crenate to serrate, and obtuse at the apex. The uppermost leaves of the plant are linear or almost entire-margined (1,3). The leaf morphology varies through the season, in the summer horseradish produces laminate leaves, while in the autumn pinnate leaves are produced (4).

Horseradish plants produce numerous fragrant flowers with 5 to 7 cm long pedicels borne on racemes that have four sepals - 2.5 to 3 mm long, broadly ovate and with a membranous white margin; four petals - white, 5 to 7 mm long and broadly obovate, and six tetradynamous stamens - 2.5 mm long, the outer ones are 1.5 mm long. The stigma is broad, round, and gently two-lobed. Horseradish bears 4 to 6 mm long, globose to obovate siliques with persistent styles on 20 mm long, upright-spreading stems (1,3). Few or no seeds are produced, maximum six seeds per pod, and when mature they are smooth and brown (4).

The root system of horseradish consists of a long, white, cylindrical root that can grow to about 60 cm in loose soils. Around the main root and near the collar of the crown, thin lateral roots are also formed. Undisturbed, the root system can reach a depth of 3 to 4 m with a lateral spread of about 1 m (3).

Figure 1 - Horseradish plant by Leonhart Fuchs (1542) (6).

1.2. Traditional Usage

The usage of horseradish as food and as medicinal herb goes back to the ancient Greeks and Romans, although it is believed that it was only until later in Middle Ages it spread from Eastern and Mediterranean areas to both north and westward. It is believed that horseradish has become popular in Europe due to its sharp spiciness that was able to cover the taste of tainted meats, as no refrigeration was available (4,5).

Since early times horseradish has been used in traditional medicine for its stimulant, rubefacient, stomachic, diaphoretic, diuretic, expectorant, antiseptic and vermifuge properties (1). Ancient Greeks used horseradish for relieve back pain and to help with digestion. They also believed that horseradish had aphrodisiac properties (3,6).

In twelfth century, Hildegard of Bingen wrote that horseradish could be used as constituents of mixes for treatment of lung disease, to cure heartache and heart diseases. Four centuries later, in Das Kreüterbuch it is claimed that horseradish should be used for the treatment of bad wounds and treatment of poisonous bites from animals as well. Besides these, it also recommended its use to treat baldness (5).

In sixteenth century, Gerard wrote that horseradish reduced pain from sciatica, relieved colic, worked as diuretic and killed worms in children. Also, it was believed to be an expectorant, and helpful in treatment of respiratory problems and relieved rheumatism by stimulating blood flow to inflamed joints (3). Although horseradish was believed to be effective against several diseases, the most common use was as a remedy for scurvy. And even though it was recommended for the treatment for scurvy since the late sixteenth/ early seventeenth centuries, it took almost 300 years to understand that vitamin C was the anti-ascorbic agent present in horseradish (a medium of three times higher than in a citrus fruit) (5).

Later in nineteenth century, Bentley and Trimen described mixtures incorporating horseradish root that were believed to treat influenza. They also described it as being a stimulant, a diuretic and diaphoretic, alongside applications as rubefacient and vesicant when applied externally. In mid twentieth century, Grieve wrote that horseradish syrup was a reliever for hoarseness and whooping cough, and to remove freckles when applied on the skin. Since then it has been used as an expectorant cough medicine, and as a cure for diseases such as rheumatism, dropsy and scurvy (3).

Nowadays there is the tradition to use horseradish in countries such as Bulgaria, Romania and Russia to treat headache, high blood pressure, bronchitis and cough (5).

In current times, horseradish has been approved as a medicine in some countries, including Germany that uses it for treatment of respiratory infections and as a supportive treatment for urinary infections, and in the United States of America where the root is the active substance from Rasapen, a drug used as an urinary antiseptic (3).

Even though horseradish was part of the culture in Europe since the Middle Ages, only later at the sixteenth century it started to be used in food at northern regions. European Jews used horseradish as a symbolic food in the ritual meal as bitter herd of Passover. The bitterness of horseradish was a reminder of the ancestors suffering from the exodus from Egypt (4).

Germany is believed to be the country that introduced horseradish as condiment or food and then it spread to England and later to the Nordic countries (5).

Germans used horseradish root as part of a sauce, cutting it into small pieces and then crushing, mixing it with salt and vinegar. The sauce was used as a side dish both for meat and fish. Horseradish leaves were also popular as a pot herb after boiled to eliminate the bitterness and the harmful substances. In later sixties, English added horseradish to their diet, becoming popular as a condiment for beef and oysters, and the leaves were also used for green mixed with other wild plants (4). Several other usages for horseradish were described everywhere in Europe, specially used as a part of sauces (5).

Nowadays, horseradish root is still popular in Europe and North America as condiment, being mixed with different sauces and served with several types of meals, from meat to salads. Also, it is incorporated as flavour in other recipes and drinks such as the Bloody Mary (5). In Bulgaria, Romania and Russia horseradish is used to flavour, help ferment and aerate pickling liquid used in several vegetables, and it is also used to preserve other vegetables for the winter such as cabbage. In Austria, horseradish is a popular side dish to Tafelspitz and Frankfurter Würstel. Also, in Poland, besides the usage as condiment it is also used as a preservative (1).

1.3. Glucosinolates and Their Degradation Products

Horseradish taste and aroma result from the presence of sulphur glucosides known as glucosinolates (7). Glucosinolates are secondary metabolites that play a major role in the defensive system of plants against insects, some food bacteria and fungi (8). Their structure consists of β-thioglucoside N-hydroxysulfates with a side chain and a sulfur-linked β-D-glucopyranose moiety (Figure 2) (2). Glucosinolates are usually classified according to the structure of the side chain in aliphatic, aromatic and indole glucosinolates that result from the different amino acid precursors (4).

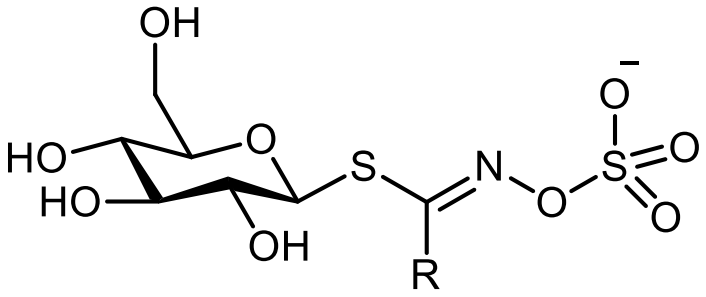

Figure 2 - Glucosinolates structure.

Glucosinolates are organic anions that are stable and soluble in water, but they degrade at temperatures above 100 °C. Below 110 °C indole glucosinolates are more unstable than aliphatic glucosinolates. The distribution of glucosinolates, both quantitative and qualitative, varies with the plants part, age, environmental factors and others (2,4).

Almost 200 glucosinolates have already been described, and their concentration in plants is around 1% of dry weight in some Brassicaceae vegetables. In black mustard seed and horseradish root their content is higher, reaching over 10% of dry weight. However, very little is known about the concentration and biochemical composition of horseradish, especially the distribution of glucosinolates in the different organs of the plants (4).

In 1980, Grob and Matile identified 30 glucosinolates in horseradish root by GC-MS and reported that the number of glucosinolates present in the horseradish is even larger. Nowadays, the scientific community is still investigating and confirming the presence of these molecules (4).

To the current day twenty glucosinolates have been identified in horseradish, some of them corresponding to the ones described by Grob and Matile. In 2012, Agneta et al. isolated 16 glucosinolates from horseradish sprouts and root by using reversed-

phase liquid chromatography coupled with electrospray ionization and a hybrid quadrupole linear ion trap and Fourier transform ion cyclotron resonance mass spectrometry (LC-ESI-FTICR MS): sinigrin, 4-hydroxyglucobrassicin, glucobrassicin, gluconasturtin, 4-methoxyglucobrassicin, glucoiberin, gluconapin, glucocochlearin, glucoconringianin, glucosativin, glucoibarin, 5-hydroxyglucobrassicin, glucocapparilinearisin or glucobrassicanapin, glucotropaeolin, glucoarabishirsutain and methylsulfonyl-oxo-ethyl-glucosinolate (9). Moreover, in 2014, the same authors reported identification of 2(*S*)-hydroxy-2-phenylethyl-glucosinolate (glucobarbarin) and/or 2(*R*)-hydroxy-2-phenylethyl-glucosinolate (epiglucobarbarin) also from horseradish. (10) In 2017, Ciska et al reported the existence of glucoraphanin, glucoraphenin and napoleiferin in horseradish (7). Even though there have been identified several glucosinolates in horseradish, sinigrin represents 64 to 86% of total glucosinolates, followed by gluconasturtiin representing between 4 and 26% (1).

Despite the defensive properties of horseradish are associated with glucosinolates, the real responsible for its activity are its breakdown products, particularly isothiocyanates. Glucosinolates hydrolysis is triggered when damage is done to the plant, and is mediated by an enzyme known as myrosinase. Myrosinase is a membrane bound enzyme that is separated from glucosinolates, which exist in vacuoles, until damage is caused to the cells (2,7).

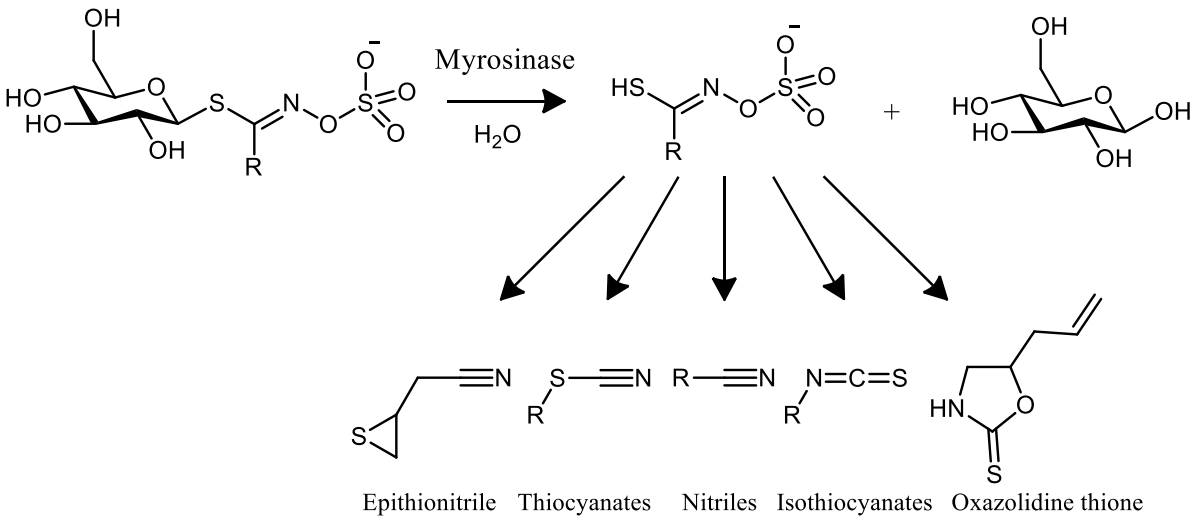

Figure 3 - Glucosinolates degradation products.

When damage is caused to the plant and myrosinase and glucosinolates are in contact, they react, and an unstable thiohydroximate-*O*-sulfonate is formed, which then rearranges to a variety of compounds that includes isothiocyanates, thiocyanates, nitriles, epithionitrile and oxazolidine thione, depending on the reaction conditions (Figure 3). The most common products are isothiocyanates (11).

Isothiocyanates consist of a -NCS group and a side chain (equal to the precursor glucosinolate). Because of the -NCS group, isothiocyanates possess electrophilic characteristics that favour reactions with nucleophilic molecules. The side chain of isothiocyanates directly influences the electrophilicity and lipophilicity of the molecule. (2) Peptides and amino acids are examples of nucleophiles found in cells, and the reaction of isothiocyanates with them could be an explanation for isothiocyanates pharmacological effects.

In 2004, D'Auria et al. identified volatile organic compounds found in fresh and after 12 hours storage horseradish samples using SPME–GC–MS (solid phase microextraction coupled with gas chromatography mass spectrometry). In fresh horseradish the main compounds were allyl isothiocyanate (AITC), 4-isothiocyanato-1-butene, and 2-phenylethyl isothiocyanate (PEITC). Allyl isothiocyanate represented 81 % of the overall compounds found. After horseradish has been cut and kept at 5ºC for 12h, the profile of volatile compounds changed, and new compounds were found. Some of them decreased their concentration and 2-phenylethyl isothiocyanate became the most abundant compound (4).

1.4. Isothiocyanates Activity

Since early days, horseradish has been added to several foods to conserve them and to be later eaten. isothiocyanates may be directly involved with the preservative activity of horseradish, since several studies have reported the antimicrobial activity of isothiocyanates. Isothiocyanates showed bactericidal activity against *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Pseudomonas corrugata*, *Salmonella typhimurium* and *Staphylococcus aureus* (12–16). Isothiocyanates also showed fungicidal activity against *Aspergillus flavus*, *Aspergillus parasiticus*, *Botrytis cinereal* and *Penicillium expansum* (16–18). Several mechanisms have been proposed for the bactericidal and fungicidal activity, although still there is not a clear understanding of it. Alongside antimicrobial

activity, allyl isothiocyanate has shown activity as insecticide against the species of *Sitophilus zeamais*, *Rhizopertha dominica*, *Tribolium castaneum* and *Liposcelis entomophilus* (19,20).

In the past decades isothiocyanates have been studied as anticarcinogenic agents, showing to be promising compounds, not only because they showed antiproliferative but also there is evidence that they are able to eliminate cancer cells. Some of the mechanisms proposed include apoptosis induction, inhibition of cell-cycle progression, angiogenesis suppression and metastasis decrease (2).

Isothiocyanates chemoprevention is usually associated with the inhibition of cytochrome P450 isoforms that are involved in carcinogens activation. It is known that most of carcinogens require enzymatic transformation by cytochrome P450 to be active. Some of the intermediates formed are electrophiles, which can react with nucleophilic sites in critical macromolecules such as DNA, RNA and proteins. In addition to the lower carcinogens activation, isothiocyanates induction of phase II enzymes and carcinogens metabolism has been associated with lower incidence of cancer (21–25).

Chronic inflammatory mediators have been associated with cancer development. Inflammation is known to favour carcinogenesis, malignant transformation, tumour growth, invasion and metastatic spread, but also it has been reported that it can limit tumour growth. The activation of transcription factors such as NF-κB, STAT-3 and HIF-1 and accumulation of tumorigenic factors, such as nitrogen monoxide, prostaglandin E2, tumour necrosis factor-α and interleukins, in tumour and microenvironment, are the linking between inflammation and cancer (26). Anti-inflammatory activity of allyl isothiocyanate and phenylethyl isothiocyanate by transcription factors inhibition have been widely studied and associated, among others, with their carcinogenic protective activity (27–31).

Studies have proven that isothiocyanates are able to arrest the cancer cell cycle in G_2/M phases, by disintegration of microtubules, specifically ubiquitination and degradation of α- and β- tubulin (32–34). Besides cell cycle arrestment, isothiocyanates have been described to trigger apoptosis which still needs a clear understanding. Mechanisms include glutathione depletion and reactive oxygen species (ROS) production (35–38), and MAPKs modulation - activation of JNK and p38, and inactivation of ERK1/2 and Akt. It has been suggested that the activation of JNK leads to Bcl-2 phosphorylation which makes the mitochondria membrane to be permeable and

to release cytochrome c and apoptosis induction factor (AIF). The released cytochrome c activates caspases 3 and 9 that together with AIF triggers apoptosis (39–44).

The development of new blood vessels from an existing vasculature – angiogenesis – is an essential mechanism for cancer development, phenylethyl isothiocyanate showed to be able to supress angiogenesis by inhibiting HIF1α translation via TSC2, suggesting that inhibition of mTORC1 is important for the phenylethyl isothiocyanate antitumor effects (45).

Metastasis is a complex process and a threat to the cancer treatment. The metastasis mechanism is uncertain and widely studied. Tumour cells spread have been associated with several factors such as cytokines, hormones, growth factors, cell adhesion molecules, and extracellular matrix proteins. Isothiocyanates were described to avoid metastasis by cancer cells (46,47).

1.5. Isothiocyanates Stability

Isothiocyanates have been reported as unstable compounds due to their electrophilic properties. It is assumed that the side chain influences the decomposition rate and route of the different isothiocyanates. Even though around 200 isothiocyanates have been identified, merely the stability of allyl isothiocyanate have been studied, probably due to the fact that allyl isothiocyanate is the main occurring isothiocyanate found in plants and the simplicity of its structure.

It was assumed that the degradation process of isothiocyanates occurs mainly by hydrolysis: R-NCS → R-OH + SCN^-, as shown in *p*-hydroxybenzyl isothiocyanate, 3-indolylmethyl isothiocyanate and 1-methoxy-3-indolylmethyl isothiocyanate. In 1969, Kawakishi and Namiki first tried to identify the decomposition products of allyl isothiocyanate in aqueous solution, suggesting its decomposition mechanism, and comparing it with the hydrolysis mechanism previously assumed. When incubating the aqueous solution of allyl isothiocyanate at 37ºC they noticed that even though the allyl isothiocyanate concentration decreased, there was not a significant increase of SCN^-, which indicated that decomposition of allyl isothiocyanate was not due to hydrolysis. They were able to identify four decomposition products (diallylthiourea, allyl allyldithiocarbamate, diallyl tetrasulfide and diallyl pentasulfide) and proposed a mechanism for its formation that included some intermediates not identified (Figure 4). Even though they proved that allyl isothiocyanate decomposition did not followed the

hydrolysis process described for *p*-hydroxybenzyl isothiocyanate, they noticed that water was an essential element for the instability of allyl isothiocyanate and its decomposition (48).

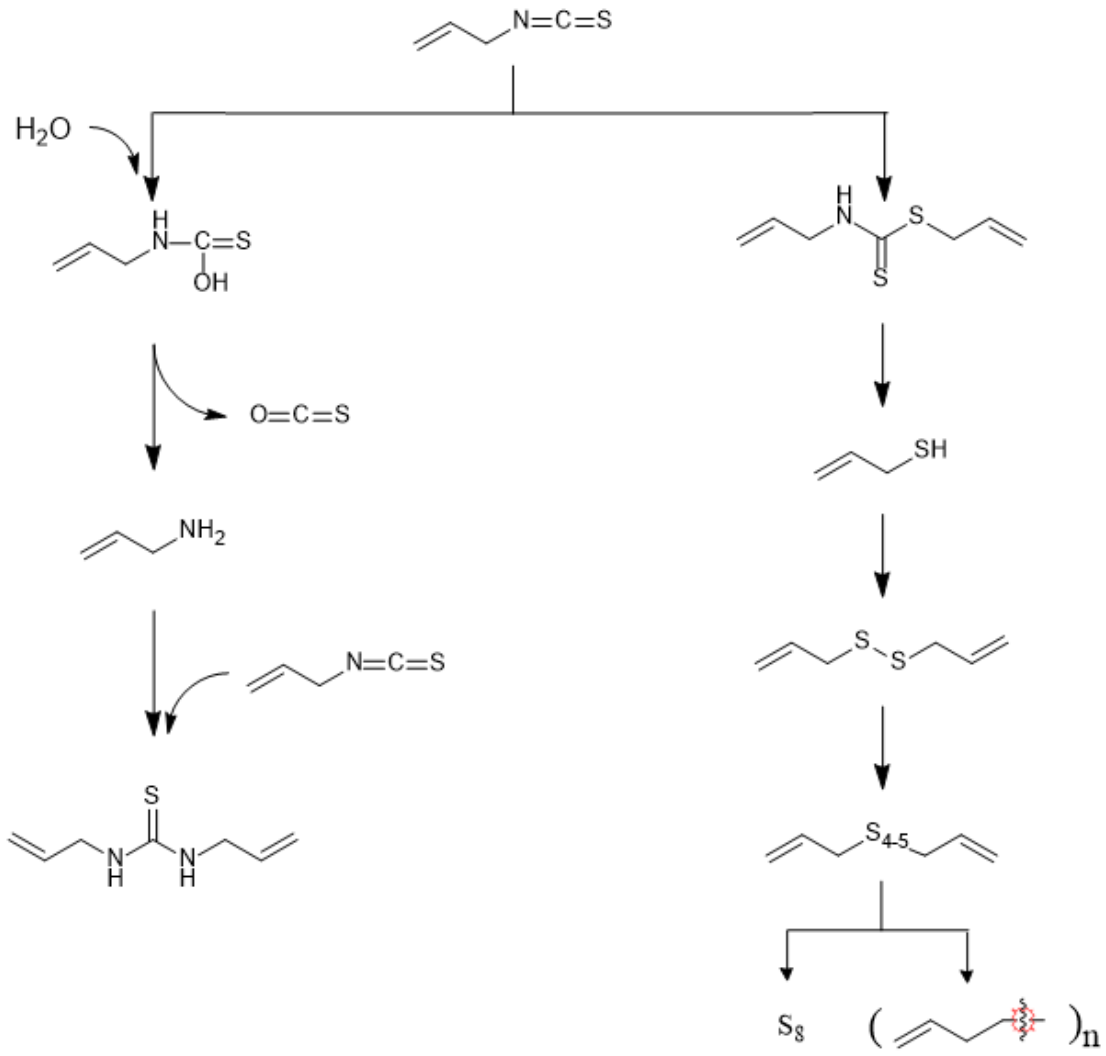

Figure 4 – Allyl isothiocyanate degradation mechanism (Kawakishi and Namiki, 48).

In 1995, Ohta et al. studied the decomposition rate of allyl isothiocyanate in aqueous solutions, testing the influence of pH, temperature and ionic strength. They noticed that ionic strength did not influence the rate of allyl isothiocyanate decomposition. On the other hand, both pH and temperature influenced the decomposition rate. Neutral and alkaline solutions promote allyl isothiocyanate decomposition, higher contents of OH^- ion accelerate the decomposition ratio. Further, temperature was described as the main factor that influenced the decomposition ratio

from the studied ones, higher temperatures promote allyl isothiocyanate degradation (49).

In 1997, continuing the study of the influence of pH and temperature in the degradation of allyl isothiocyanate, Pecháķĉek et al. analysed over time (1h20) the degradation products of aqueous solutions of allyl isothiocyanate at pH 4, 6 and 8, baked at 20, 40 and 80ºC. Ten degradation products were identified, including two compounds previously isolated by Kawakishi and Namiki and three that were described as potential intermediates by the same authors, in addition three new decomposition products were found. Taking in account the temperature, there was not found significant differences among the compounds identified at the three temperatures studied, only quantitative wise, where 80ºC provide higher amounts of degradation products. The decomposition ratio of allyl isothiocyanate in solutions with pH 6 and 8 were similar; on the other hand, the solution with pH 4 had lower decomposition ratio. In the study they also provided several mechanisms that could explain the formation of the different degradation products (50).

In 1998, Chen and Ho studied the thermal degradation of allyl isothiocyanate in aqueous solution, cooking it at 100ºC for one hour. They identified nine volatile degradation products and one non-volatile product. They proposed a possible mechanism for the formation of the volatile products, taking in account the mechanism proposed by Kawakishi and Namiki since they were not able to identify some degradation products previously identified at the study conditions, these mechanisms included radical and oxidation reactions. Once again, they reinforced that higher values of pH promoted allyl isothiocyanate degradation (51).

Chen et al, while trying to use the previous conditions as described before to identify both volatile and non-volatile degradation products of phenylethyl isothiocyanate, were not able to find any volatile degradation compounds and only found diphenethylthiourea as a non-volatile degradation product. Besides this study, there were no other studies found to investigate phenylethyl isothiocyanate stability and degradation products (52).

Besides using lower temperatures and low pH values for improving isothiocyanates stability in water solutions there have been studies performed using different strategies. Among them, positive results have been found when isothiocyanates were complexed with cyclodextrins (53,54) and by integrating it in an oil-in-water nano-emulsion (55).

1.6. Lamiaceae Family

The Lamiaceae family has over than 7000 species in 236 genera. Most species are shrubby or herbaceous and rarely trees are found. This family has great economic value, as it contains a great number of horticultural species, several of them used as condiments. They can be found all over the globe, although tropical (savannas) and temperate (Mediterranean region) areas are favourable (56). Besides the culinary uses, several species have been used for treating asthma, cramping, diarrhea, indigestion, infectious diseases, muscle pain, nausea and rheumatism. Reports point out that Lamiaceae species, among others, exert anti-inflammatory, antioxidant, antibacterial, antifungal, and antiviral effects (57).

Lamiaceae is rich in phenolic compounds (phenols and polyphenols) that denote a major group of plant secondary metabolites. Phenols have at least one phenolic ring formed by a hydrophobic aromatic nucleus and a hydrophilic hydroxy group, which can be involved in hydrogen-bond formation (56). Phenolic compounds were identified in several classes of compounds which include phenolic acids, flavonoids, phenolic diterpenes and volatile oils (58). Being redox-active compounds, plant phenols can also act either as antioxidants or as pro-oxidants. The antioxidant behaviour depends on the number of hydroxy substituents, their mutual position and the binding site on the aromatic ring (56). This effect is the result of various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, decomposing peroxides, and/or singlet and triplet oxygen quenching capacity (58,59).

1.7. Objectives

Isothiocyanates are valuable compounds that can have a wide future application, although their stability is still a problem that scientific community needs to overcome. The main goal of this study was to investigate the influence of aqueous extracts from different plants from Lamiaceae family in the stability of the main isothiocyanates of horseradish. The plants used were *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia officinalis*, *Saturejae montanae* and *Thymus vulgaris*. In order to achieve the main goal, several small tasks were defined, including the establishment of a viable method to obtain and quantify the isothiocyanates from the solutions and have a clear picture of the main isothiocyanates found in the plant material used.

The method chosen for extraction of isothiocyanates was solid phase extraction (SPE). SPE was selected due to its advantages compared to the other methods described for isothiocyanates obtainment – liquid-liquid extraction, soxhlet and hydro-distillation. Among the advantages it is included: the need of low amounts of organic solvents; the short extraction time; no temperature required; and most important the not need of a follow clean-up before the chromatographic analysis.

2. Materials and Methods

2.1. Chemicals, Plant Material, Reagents and Solvents

Origanum vulgare – Herba Origani (vulgaris) CS, *Thymus vulgaris* – Herba Thymi in fol. PhE, *Rosmarinus officinalis* – Fol. Rosmarini, *Saturejae montanae* – Herba Saturejae montanae CS, and *Salvia officinalis* – Folium Salviae officinalis CS were obtained from Kottas Pharma (Vienna, Austria). *Armoracia rusticana* - horseradish, roots were obtained from a local farmers market and identified by Professor Doctor Franz Bucar.

Allyl isothiocyanate 95%, phenethyl isothiocyanate 99%, 3-phenylpropionitrile 99% and 2,2-diphenyl-1-picrylhydrazyl 90%, were obtained from Sigma-Aldrich. Benzyl isothiocyanate 97% was obtained from Fluka. Folin-Ciocalteu's phenol reagent was obtained from Merck KGaA. Quercetin and gallic acid monohydrate were obtained from Carl Roth GmbH + Co. KG.

Methanol, dichloromethane, ethanol and acetonitrile were obtained from VWR Chemicals. Deionized water was produced by a PureLab, Prima system, while ultrapure water was produced by a EASYPure RF, Barnstead, purification system.

2.2. Assessment of Solid Phase Extraction

To determine the best method for SPE extraction of isothiocyanates, experiments using two brand-name commercial columns was carried out - Isolute® C18-EC (500 mg), Biotage, and Oasis® HLB 6cc (500 mg), Waters. Conditioning, washing, and elution procedures were those generally recommended by manufacturers. After successive conditioning with 5 mL of methanol and 5 mL of water for Oasis® columns and with 3 mL of methanol and 3 mL of water for Isolute® columns, cartridges were loaded with 25 μL of an isothiocyanates working solution in ethanol (allyl isothiocyanate, benzyl isothiocyanate and phenylethyl isothiocyanate 10 mg/mL) diluted in 10 mL of water (25 μL + 10 mL). They were then washed with 10 mL of a water/methanol solution (95:5; v/v). Finally, isothiocyanates were eluted, first, with methanol (three elution steps of 2 mL each), then with dichloromethane (two elution steps of 2 mL each). With the selected SPE sorbent, an alternative washing procedure was also used which consisted of 10 mL of water, in addition several elution alternative washing procedures were tried to determine the optimal procedure. With each elution procedure collected, 780 μL of elute were supplemented with 20 μL of 3-

phenylpropionitrile solution at 4 mg/mL and analysed by GC-MS, methanolic elution fractions were also analysed by HPLC.

2.3. HPLC Analysis

HPLC analysis was performed using a Merck-Hitachi D-7000 equipment, equipped with a L-7100 pump, a L-7455 diode array detector, a L-7250 programable auto sampler and a Jetstream II Plus column thermostat.

For analytical procedures, a Zorbax Eclipse Plus C18 (150 x 2.1 mm, 3.5 μm) was used. Elution was carried out at a flow rate of 0.250 mL/min using a solvent gradient system of water/acetonitrile. The system started at 50:50 and was linearly increased to 58% of acetonitrile in 10 min, followed by linear increase to 80% of acetonitrile in further 10 min. The temperature of the oven was kept at 35 ºC and eluate was monitored at 229 nm.

For isolation procedures, a LiChrospher® 100 RP_{18} (5.0 μm) LiChroCART® (250 x 4.0 mm) was used. Elution was carried out at a flow rate of 1 mL/min using an isocratic solvent system of 42:58 water/acetonitrile for 16 min. The temperature of the oven was kept at 35 ºC and eluate was monitored at 229 nm.

2.4. Standard Calibration

Allyl isothiocyanate and phenylethyl isothiocyanate contents were determined by HPLC. Six different concentrations of allyl isothiocyanate (50, 100, 150, 200, 250 and 400 μg/mL) and phenylethyl isothiocyanate (10, 30, 60, 100, 150 and 200 μg/mL) were measured, in triplicate, at wavelength of 240 and 210 nm, respectively, and afforded the calibration lines.

$$\text{AITC } (\mu g/mL) = 6318.4\ x + 303.59,\ R^2 = 0.9997 \qquad (1)$$

$$\text{PEITC } (\mu g/mL) = 49803\ x + 81290,\ R^2 = 0.9995 \qquad (2)$$

2.5. GC-MS Analysis

Gas chromatography was performed using an Agilent 7890A gas chromatograph, equipped with a 7683B automatic liquid sampler, a 7683B injector and a 5975C VL mass detector. A fused silica capillary column HP-5MS (30 m x 250 μm x

0.25 μm, Agilent) was used. Two programs were used differing only in the injection mode, one being splitless, and one using a 20:1 split ratio. The GC oven was temperature-programmed for 50 ºC for 1 min, followed by an increase from 50 to 180 ºC at a rate of 3 ºC/min. The carrier-gas used was helium (5.6) with a flow rate of 1 ml/min. The temperature of injection was 260 ºC, interface 280 ºC, detector EI-MS, 70 eV, 230 ºC for ion Source and 150 ºC for quadrupole. Injections of 1μL were used.

2.6. Aqueous Extract Preparation

For the studied Lamiaceae species, 10 g of grinded plant material was extracted with 100 mL deionized water and heated under reflux for 15 minutes. After cooling, the extract was centrifuged at 3000 rpm for 15 mins (Centrifuge, Eppendorf 5810R) and filled up in a volumetric flask to 100 mL with deionized water.

2.7. Combination with Horseradish

4 g of horseradish root was cut to small pieces and then was homogenized with 76 g of aqueous extract in a blender. The mixture was put out of the blender and left at room temperature for 2 hours. After centrifugation (3000 rpm, 15 mins), 10 ml were extracted for isothiocyanates by SPE. The methanolic extracts were finally analysed by HPLC-PDA for quantification and by GC-MS for identification of isothiocyanates.

2.8. Paste Preparation

25 g of horseradish root, previously homogenised in a blender, was mixed with 1 g of grinding plant material and 1 ml of water in a mortar. 25 g of horseradish root mixed with 1 ml of water was used as a comparison. The content of isothiocyanates was determined at 0, 24 and 48 hours, by diluting the amount of paste in 20 ml of water corresponding to 500 mg of paste/1 ml of water, the sample was left at room temperature for the different time periods, followed by filtration. 10 ml of the filtrate was subjected to SPE extraction, and the methanolic eluate was analysed by HPLC.

2.9. Degradation Products Identification

40 g of horseradish was mixed with 100 ml of water in a blender, and left at room temperature being centrifuged 24h after. At 24 and 48h, 10 ml were subjected to SPE, and the methanolic eluates were analysed by GC-MS and HPLC.

From the 48h methanolic extract, one degradation product was isolated using HPLC, being afterwards analysed by GC-MS.

Allyl and phenyl ethyl isothiocyanates water solutions were also prepared and left at room temperature for 48h for later degradation products search.

2.10. Aqueous Extract Residue

The aqueous extract residue was determined recurring to Sartorious Moisture Analyzer MA45. The results were expressed in % (w/w: residue/ aqueous extract).

2.11. Determination of the Total Phenolic Content

The total content of phenolic compounds was determined using Folin–Ciocalteu's reagent and the method of Cai et al. (60). This reagent reacts with all oxidable OH groups present in the sample. To determine the total phenolic content, gallic acid was selected as the external standard for generating the calibration curve. Five different concentrations of gallic acid (1000, 250, 125, 62, and 31 μg/mL) were used.

0.5 mL sample (previously diluted to concentration of 5% residue/ aqueous extract) was diluted with 7 mL of water and vortexed for 5 s. Subsequently, 0.5 mL Folin–Ciocalteu's reagent was added and the solution was mixed again for 5 s. After 3 min of incubation at room temperature in the dark, 1 mL of saturated Na_2CO_3 solution was added. The mixture was diluted up to a volume of 10 ml with water and incubated for 1 h on a drying oven (Heraeus Instruments, FunctionLine T6) at 25 °C. The absorption was measured using a microplate reader (Hidex Sense platereader, HVD Life Science Vertriebs GmbH) at 725 nm, and it was used a 96-wel plate with lid (CytoOne ®, polystyrene, flat bottom, tissue culture treated, sterile, individually wrapped, 50/case). The total phenolic content of the extracts was calculated using the calibration curve of gallic acid. The results were calculated on the plant extracts (60,61).

2.12. DPPH Assay – Radical Scavenging Capacity

In vitro non-specific radical scavenging capacity of the plant extracts was determined using 1,1-diphenyl- 2-picrylhydrazyl (DPPH) according to the method of Schneider (62,63). Methanolic extracts (prepared by heating 1 g of plant material with 10 ml of methanol for 15 min under reflux, filtering and bringing the filtrate in a

volumetric flask to 10.0 ml with MeOH) were diluted with MeOH to give 5 concentrations: 10.0 mg/ml, 3.3 mg/ml, 1.1 mg/ml, 0.3 mg/ml, 0.1 mg/ml, and all measurements were conducted in triplicate. In a microtiter plate to 50 µl extract dilutions 150 µl of DPPH solution (50 µM DPPH in MeOH) were added. DPPH solution should be protected from light. The inhibition ratio was determined spectrophotometrically at 517 nm after 30 min of incubation in the dark, subtracting the corresponding background values (50 µl diluted extract + 150 µl MeOH). As positive control quercetin at five concentrations in methanol (0.2 µg/ml; 1 µg/ml; 5 µg/ml, 25 µg/ml, 125 µg/ml were used (50 µl quercetin solution + 150 µl DPPH solution).

Inhibition percentage was calculated using the following equations:

$$abs_{control} = abs_{MeOH+DPPH} - abs_{blank} \quad (3)$$

$$\text{inhibition percentage} = [(abs_{control} - abs_{sample})/abs_{control}] \times 100 \quad (4)$$

Results were expressed as IC_{50} values (concentration of inhibitor to cause a half-maximum inhibition of the reaction) in relation to control using Microsoft Excel for calculation (62,63).

3. Results and Discussion

3.1. Method Optimization

Isothiocyanates are structural diverse and have different chemical properties, including their polarity. Having highest recovery rate of the isothiocyanates in mind, two different type of sorbents were studied (64). Using a standard solution containing allyl isothiocyanate, benzyl isothiocyanate and phenylethyl isothiocyanate, the recovery rate for isothiocyanates were compared between Oasis HLB, a divinylbenzene polymeric sorbent, and Isolute C_{18}-EC, a silica-based C_{18} sorbent. By HPLC and GC-MS analysis of the eluates it was possible to notice that Isolute C_{18}-EC sorbent had higher recovery rates than the Oasis HLB, therefore Isolute C_{18}-EC sorbent was selected for the following studies. The optimized method was determined for Isolute C_{18}-EC, and it consisted of 3 ml of methanol and 3 ml of water for conditioning, 10 ml of sample, washing with 10 ml of water, and elute with 3 ml of methanol. To achieve this procedure several conditions were evaluated, among them: the washing process was tested with water and with water/methanol solution (95:5; v/v), the washing with water/methanol solution had lower recovery rate for allyl isothiocyanate than water; methanol and ethanol were tested as elution solvents, and it was found that when ethanol was used as an eluent it had lower recovery rates than methanol; and finally, the volume of eluent was determined to be 3 mL as the optimum (data not shown).

3.2. Isothiocyanates Identification

GC-MS and HPLC analysis of a solution containing a mixture of allyl isothiocyanate, benzyl isothiocyanate and phenylethyl isothiocyanate allowed the determination of the retention times for individual isothiocyanate and by analysing a solution of horseradish in a thyme aqueous solution (1 g horseradish/ 20 g solution), allowed to establish the allyl and phenylethyl isothiocyanates as the main isothiocyanates found in the horseradish root as described in the literature (65,66). In the GC-MS chromatogram also thymol could be detected, that is a monoterpene phenol found in thyme but not in horseradish (Figure 5).

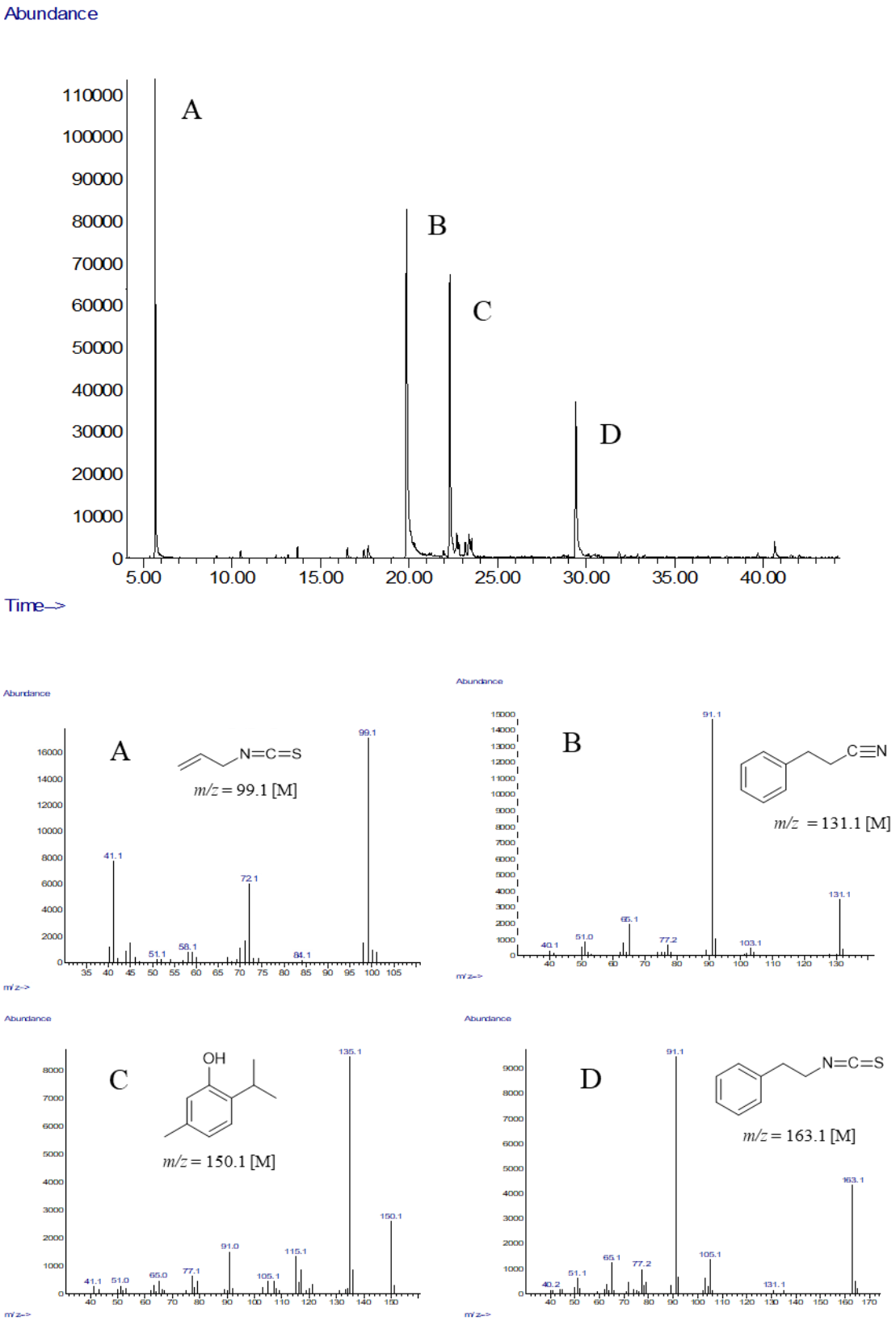

Figure 5 – GC-MS total ion chromatogram and EI mass spectra. A- Allyl isothiocyanate; B- Benzenepropanenitrile; C- Thymol; D- Phenethyl isothiocyanate.

3.3. Recovery Rate

Using standard solutions of allyl isothiocyanate (concentrations between 50 and 400 μg/mL) and phenylethyl isothiocyanate (concentrations between 10 and 200 μg/mL) calibration curves were determined (Appendix 1).

Submitting two different standard solutions with concentrations of 10 and 20 mg/mL to SPE, the recovery rate was determined in triplicate. The results showed that the yield was different according to the isothiocyanate, that could be explained by difference in chemical structure (64). Allyl isothiocyanate recovery rate can be considered 100%, or very close to it, on other hand, phenylethyl isothiocyanate recovery rate was lower than allyl isothiocyanate, and its recovery decreases with the increase of the initial concentration, i. e. 92.5 % for 10 mg/mL and 82.6 for 20 mg/mL (Table 1).

Table 1 – Isolute C_{18}-EC recovery rate of Allyl and Phenylethyl isothiocyanates.

Concentration (mg/mL)	Recovery Rate (%)	
	Allyl isothiocyanate	Phenylethyl isothiocyanate
10	99.5±1.7	92.5±2.5
20	110.0±5.6	82.6±2.2

3.4. Aqueous Solutions of Studied Plants on Stabilizing Isothiocyanates

Several aqueous extracts from the studied Lamiaceae species were mixed with horseradish in proportion 1:19 (horseradish/aqueous extract) to evaluate its influence in the degradation of isothiocyanates. The allyl and phenylethyl isothiocyanates content in the samples was determined at 24 and 72 h after sample preparation analysing the methanolic elute obtained after the samples being submitted to SPE.

Considering the allyl isothiocyanate content to be similar between the samples at 0 h, the content of allyl isothiocyanate at 24 h presented some differences between them, particularly the satureja solution that presented the lowest concentration among the samples. At 72 h the concentration of allyl isothiocyanate was distinct between the samples, and was possible to differentiate the influence of the aqueous extracts on the degradation of allyl isothiocyanate. Rosemary aqueous solution not only exhibited the higher content of allyl isothiocyanate, but also the degradation ratio was the lowest.

Oregano and thyme aqueous solutions followed the rosemary sample, both showing similar content and the degradation ratio between them. Sage and satureja presented the lowest allyl isothiocyanate content and the highest degradation ratio (Figure 6).

The content of phenylethyl isothiocyanate in the samples at 24 h was similar between them. Oregano sample exhibited the highest concentration. At 72 h the phenylethyl isothiocyanate concentration in the samples differed. Rosemary and oregano samples displayed similar content in phenylethyl isothiocyanate, however the degradation ratio was lower in the rosemary sample. Phenylethyl isothiocyanate content in sage and thyme solutions followed. phenylethyl isothiocyanate was detected in the satureja sample, but its content was out of quantification limit (Figure 7).

Considering the obtained results, oregano and rosemary were selected as the most promising samples on minimizing the degradation of isothiocyanates and the following studies were performed mainly with these plants.

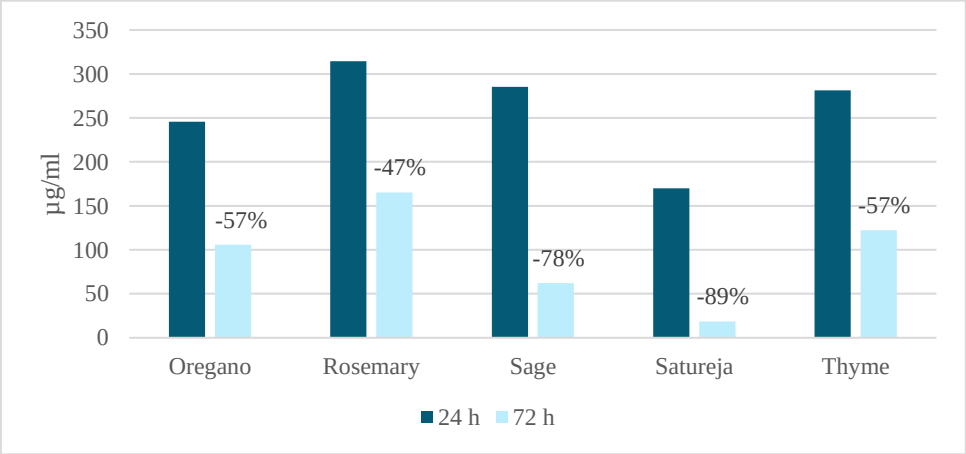

Figure 6 – Determination of allyl isothiocyanate concentration of the aqueous solutions at 24 and 72h.

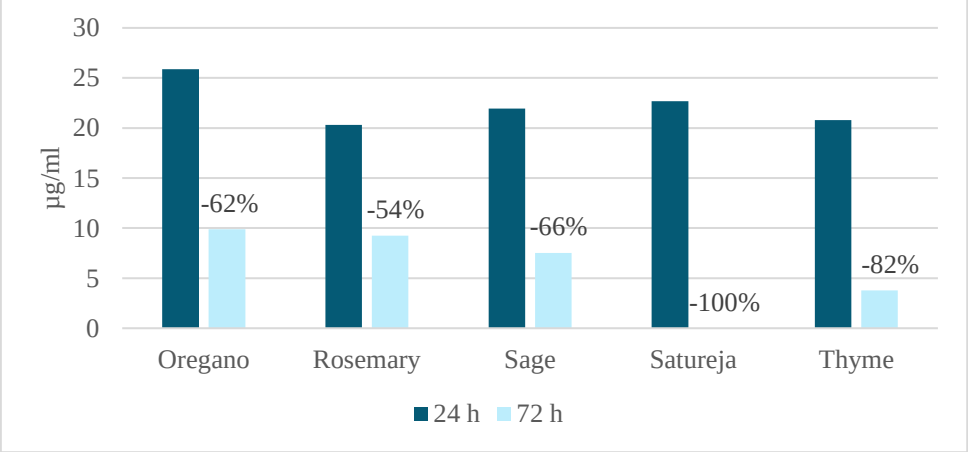

Figure 7 - Determination of phenylethyl isothiocyanate concentration of the aqueous solutions at 24 and 72h.

3.5. Aqueous Extract Concentration Influence on Isothiocyanates Stability

Aiming at understanding how the concentration of the aqueous extract would influence the stability of isothiocyanates and their degradation, four different aqueous extracts of oregano and rosemary with different concentrations (5, 10, 15 and 20 g plant material/ 100 ml water) were used. An aqueous solution containing the same content of horseradish was used as comparison. The isothiocyanates content was determined at 2, 24 and 72 h. This experiment was conducted with a new plant material resulting in a different isothiocyanates content. The isothiocyanates content in the new plant material was lower than in the previous one, the difference between them was high enough to influence the experiment planning, as the concentration at 72 h in the solutions was too low to be measured. Having this in mind, only the 2 and 24 hour results were taken into account, and the following experiments were planned using a maximum of 48 h incubation time.

Allyl isothiocyanate content at 2 h differed among the samples. It was possible to notice that the samples containing plant extracts had higher concentration of allyl isothiocyanate than the sample prepared with water. Moreover, oregano samples also presented higher amount of allyl isothiocyanate than the rosemary sample. This difference in the content could be explained by the fact that rosemary samples were submitted to SPE after the samples containing oregano. The difference found between the allyl isothiocyanate content on the different extract concentrations could be explained by different HPLC analysis times, the lowest concentration samples being analysed first (Figure 8).

At 24 h, differences in both concentration and degradation ratio among the samples could be observed. Allyl isothiocyanate concentration in the water solution was in the same range of the samples containing plant extracts, which provided the sample with the lowest degradation ratio. Analysing the content of allyl isothiocyanate in oregano samples, the sample with 15 mg oregano/100 mL water extract presented the highest concentration of allyl isothiocyanate and lowest degradation ratio among the oregano samples, followed by 10, 5 and 20 mg oregano/100 mL. On the other hand, rosemary samples behaved in a different matter. Allyl isothiocyanate content was higher at the sample with 5 mg rosemary/100 mL, followed by 20, 25 and 10 mg plant/100 mL, but the degradation ratio didn’t follow the content of allyl isothiocyanate in the samples,

instead the 20 mg plant/100 mL had the lowest degradation ratio. However, it has to be taken into account that the allyl isothiocyanate starting concentration could have been different as it was not possible to handle all samples and perform all analyses simultaneously (Figure 8).

The pattern of content of phenylethyl isothiocyanate was completely different from allyl isothiocyanate. Comparing phenylethyl isothiocyanate concentrations at 2 h, samples containing oregano extracts had higher level than water and rosemary samples (Figure 9).

At 24 h, on the contrary of allyl isothiocyanate the content of phenylethyl isothiocyanate in the water sample was the lowest and its degradation ratio the highest. The concentration of phenylethyl isothiocyanate in oregano samples was directly proportional to the extract concentration, although the sample containing the 20 g/ 100 mL extract showed a higher degradation ratio than the 15 g/ 100 mL sample. Taking these results into account, including those of allyl isothiocyanate, the sample containing 15 g/ 100 mL extract was determined to be the most efficient in stabilizing isothiocyanates (Figure 9).

For rosemary samples, there was not a direct correlation between phenylethyl isothiocyanate content and the concentration of the aqueous extracts and the obtained results were comparable to those obtained for the allyl isothiocyanate. Aiming at choosing the concentration that would be the best for stabilizing isothiocyanates two approaches could be considered. On one hand, the sample with the higher concentration extract (20g / 100 mL) could be picked, because ultimately it had the lowest degradation ratio, for both allyl and phenylethyl isothiocyanates. On the other hand, the one with lowest concentration could be picked, taking in account that the difference in the isothiocyanates content is not that significant. It would be necessary to have a different approach to determine what should be the exact concentration of aqueous extract to improve the stability of the compounds. A proposed method could pass by the preparation of an aqueous extract with a higher concentration of plant material, dilute it four to five times, and then study the influence in the compound stability. Furthermore, the time between sample preparation (including control samples) and HPLC analysis should be equalized.

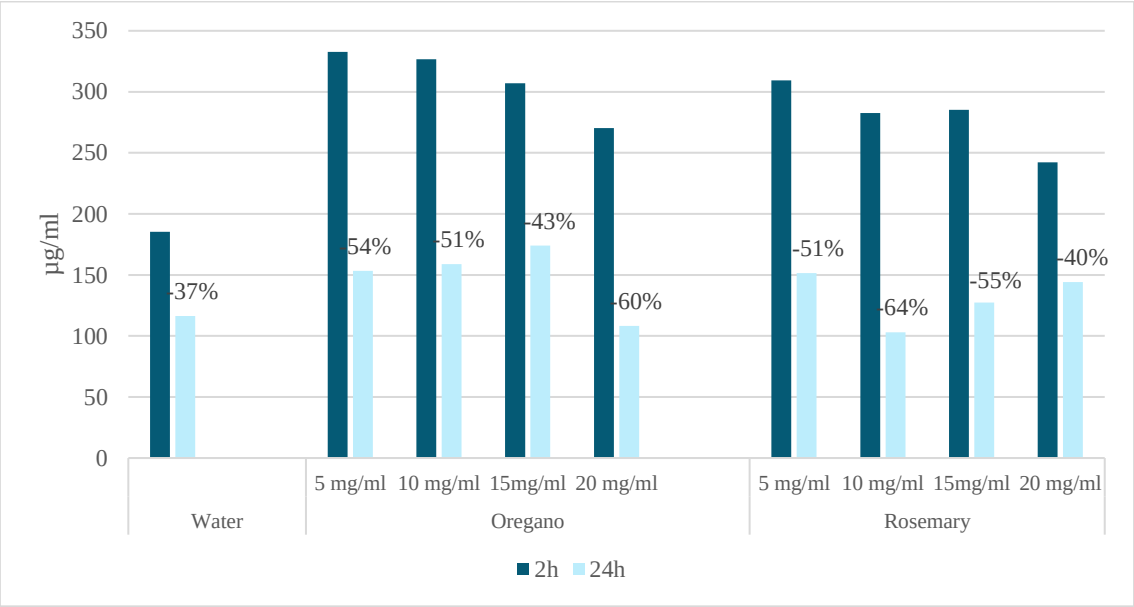

Figure 8 - Determination of allyl isothiocyanate concentration of aqueous solutions with different concentrations of oregano and rosemary at 2 and 24h.

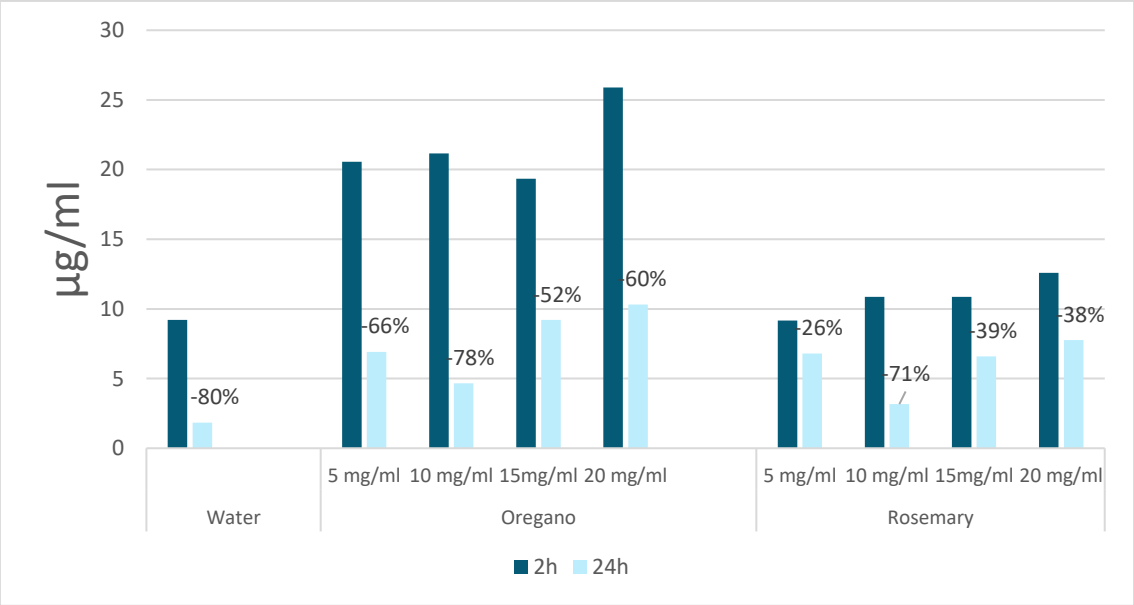

Figure 9 - Determination of phenylethyl isothiocyanate concentration of aqueous solutions with different concentrations of oregano and rosemary at 2 and 24h.

3.6. Dried Plant Influence on Stabilizing Isothiocyanates

Continuing to study the influence of oregano and rosemary on the stability of the isothiocyanates from horseradish, a new approach was tempted. In this approach, the water content was minimized to almost the minimum. For that purpose, horseradish was homogenized in a blender, and then it was mixed with both oregano and Rosemary grinded plant material in a mortar, with 1 ml of water. The same amount of horseradish was mixed with 1 ml of water to be used as a comparison. The isothiocyanates content was determined at 2, 24 and 48 h.

At 2 h time, allyl isothiocyanate content was higher in the sample containing only horseradish compared to the ones that were mixed with oregano and rosemary. This fact could be explained by the sample preparation, since that in comparison to the water sample it took some time to get a homogenous paste with the grinded plant materials (Figure 10).

It was possible to observe that over time the content of both allyl and phenylethyl isothiocyanates in the pure horseradish samples decreased. Conversely, in the Rosemary and Oregano samples mixed with horseradish the content of isothiocyanates maintained constant. At 24 h the Oregano sample showed a low concentration of allyl isothiocyanate, but it could be explained by some error of the sample manipulation (Figures 10 and 11).

This study not only permitted to confirm that the water is a major influence in the isothiocyanates degradation as described in the literature, (48) but also that both plants could minimize the degradation of isothiocyanates.

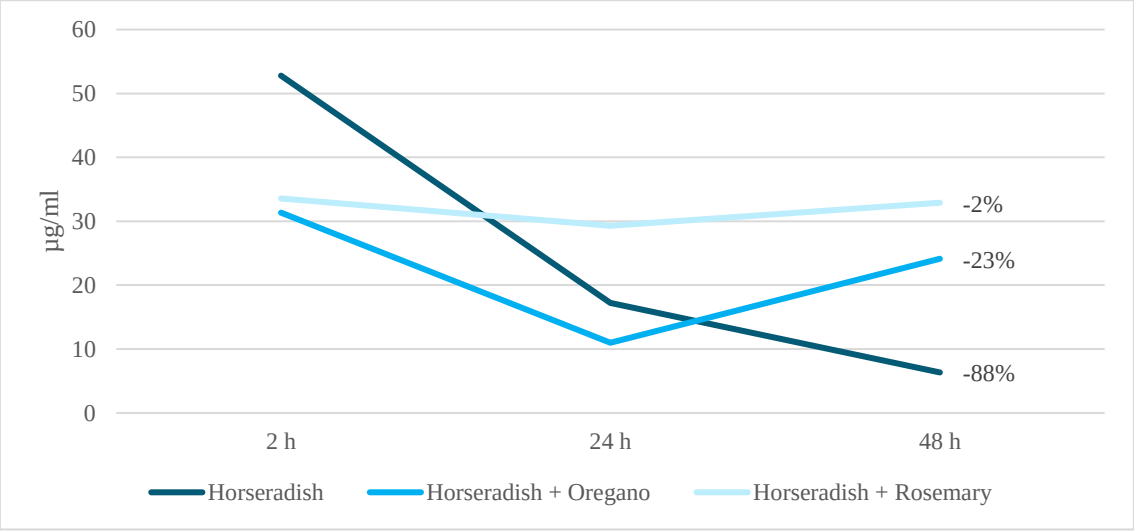

Figure 10 – Determination of allyl isothiocyanate content in paste.

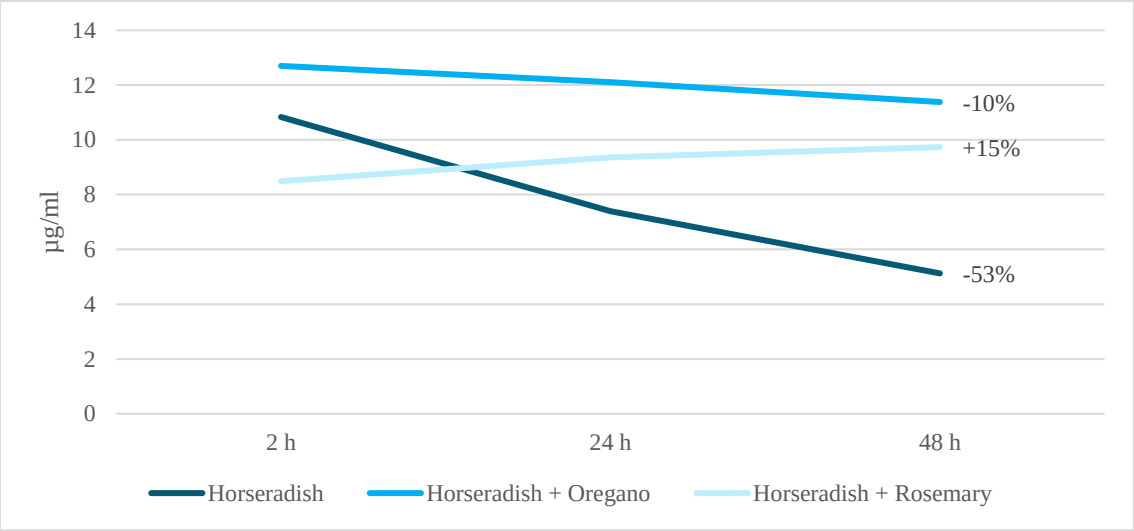

Figure 11 – Determination of phenylethyl isothiocyanate content in paste.

3.7. Total Phenolic Content and DPPH Scavenging Capacity

The yields of the aqueous extracts (w/w) were 1.17 % for oregano, 1.55 % for rosemary, 1.10 % for sage, 0.98 % for satureja and 0.85 % for thyme. The total phenolic contents of the extracts were determined to be: oregano 85 mg, rosemary 82 mg, salvia 47 mg, satureja 51 mg and thyme 43 mg gallic acid equivalents (GAE)/ g.

In DPPH assay, where the ability of scavenging against radicals was tested, sage and rosemary showed the highest activity followed by oregano, thyme and satureja (Table 2). The results obtained are in accordance to those reported in literature (57).

Table 2 - Radical scavenging activities of the studied Lamiaceae plants aqueous extracts against DPPH.

	Origanum vulgare	*Rosmarinus officinalis*	*Salvia officinalis*	*Saturejae montanae*	*Thymus vulgaris*
IC_{50} (µg/ml)	5.3	2.6	2.5	7.0	6.5

When comparing the total content of phenols with the capacity of reduction of isothiocyanates degradation by the different plants, some relationships could be observed. It is possible to notice that the aqueous extracts presenting the higher phenolic content (oregano and rosemary) were also the ones picked as the best contenders for decrease of isothiocyanates degradation. Knowing that phenolic compounds are antioxidant and that their main antioxidant mechanism is to be the scavenging of different types of reactive oxygen and nitrogen species, (57) the DPPH test was

performed. Although DPPH result for rosemary was consistent with its reduction of isothiocyanates degradation, both Sage and oregano were not, which led to think that probably there is a not a clear relation between the radicals scavenging and the isothiocyanates stabilization, and other mechanism could be responsible.

3.8. Degradation Products

Trying to search for allyl and phenylethyl isothiocyanates degradation products, water solutions were prepared and left at room temperature for 48h. After subject to SPE, from the allyl isothiocyanate solution was possible to identify methyl cyclopropylcarbamate, on the other hand benzene acetaldehyde and benzyl thiocyanate was identified from phenylethyl isothiocyanate solutions. Besides the search for degradation products in allyl and phenylethyl isothiocyanates water solutions, potential degradation products were also investigated in a horseradish water solution. It was possible to identify methyl cyclopropylcarbamate, indole, 1-isothiocyanato-3-(methylthio)-propane, iberin, indole-3-ethanol and indole-3-acetonitrile (Figure 12). From the horseradish solution a compound was isolated by HPLC that increased over time and was identified as methyl cyclopopylcarbamate.

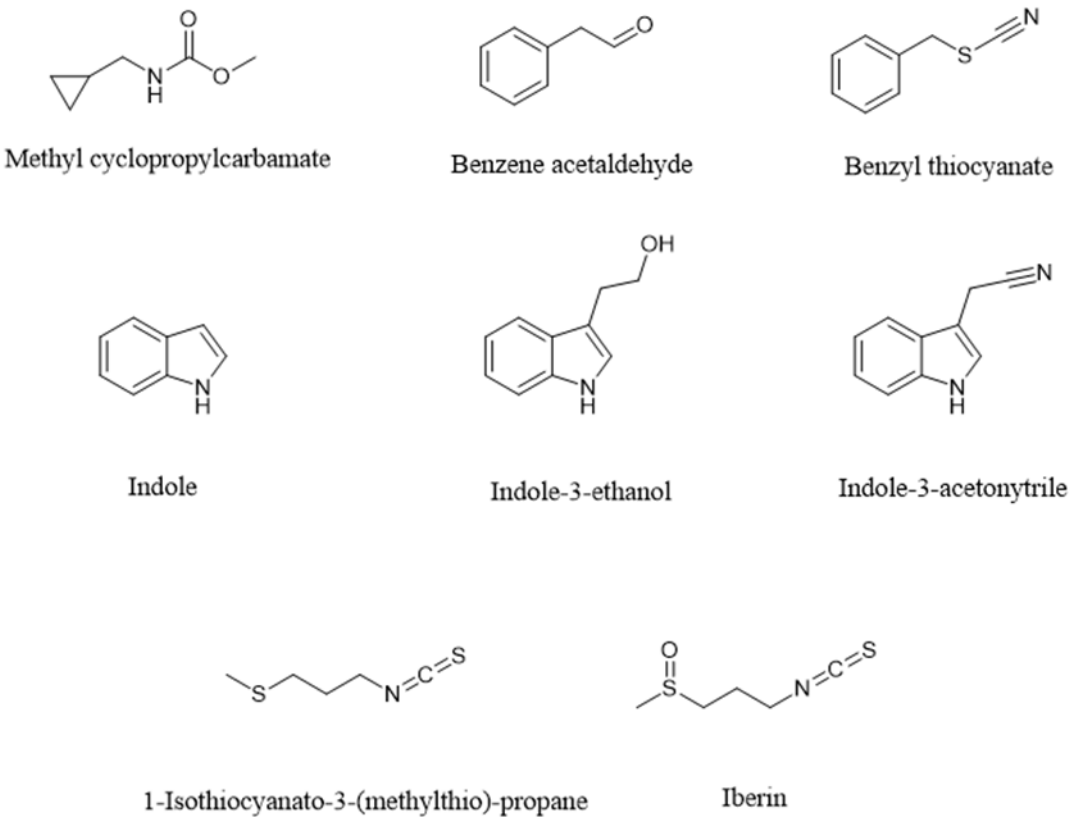

Figure 12 - Possible degradation products identified.

4. Conclusions

This work focused on the search for a novel approach to stabilize horseradish isothiocyanates and prevent their degradation in aqueous solution. Although there are several studies reporting on isothiocyanates activity as antimicrobial and chemoprotective, there are just a few studies on their degradation process and how to stabilize them. Known to be antioxidant, Lamiaceae plants appeared to be potential adjuvants for isothiocyanates stabilization.

In this study aqueous extracts of five Lamiaceae medicinal plants were evaluated for their influence on isothiocyanates degradation. The difference on the results obtained among the different plants suggested that there could have some influence on the degradation of isothiocyanates. Oregano and rosemary aqueous extracts showed to be the most promising on the decrease of isothiocyanates degradation, followed by sage and thyme and finally by satureja. Further studies performed with oregano and rosemary provided information corroborating the hypothesis that the studied Lamiaceae plants could be helpful for isothiocyanates stabilization.

When trying to understand if the antioxidant activity was related with the decreased of the isothiocyanates degradation, it was found that both Oregano and Rosemary aqueous extracts had the highest total phenol content, which could be related to the stabilization of isothiocyanates. In addition, the capacity of scavenging free radicals was tested, and it was found that Sage and Rosemary presented the highest capacity. These results indicated that this mechanism may not have a significant (or pronounced) influence in isothiocyanates degradation.

Being the initial study on the influence of Lamiaceae plant extract on the degradation of isothiocyanates there is still a large amount of work to be performed. This future work should focus on the influence of the concentration of the aqueous extract on the stabilization of isothiocyanates; on continuing to study the difference between the isothiocyanates degradation in aqueous solutions with and without Lamiaceae plant; and on the mechanism of Lamiaceae plants in decreasing isothiocyanates degradation. Other factors to be investigated should include temperature, especially the influence of lower temperatures.

5. References

1. Lim TK. Edible Medicinal And Non-Medicinal Plants. Vol. 9. Springer Science; 2013. 746-760 p.
2. Nguyen NM, Gonda S, Vasas G. A Review on the Phytochemical Composition and Potential Medicinal Uses of Horseradish (*Armoracia rusticana*) Root. Food Rev Int. 2013; 29 (3): 261–75.
3. Shehata A, Mulwa RMS, Babadoost M, Uchanski M, Norton MA, Skirvin R, et al. Horseradish: Botany, Horticulture, Breeding. Janick J, editor. Vol. 35, Horticultural Reviews. John Wiley & Sons, Inc.; 2009. 221-261 p.
4. Agneta R, Möllers C, Rivelli AR. Horseradish (*Armoracia rusticana*), a neglected medical and condiment species with a relevant glucosinolate profile: A review. Genet Resour Crop Evol. 2013; 60 (7): 1923–43.
5. Wedelsbäck Bladh K, Olsson KM. Introduction and use of horseradish (*Armoracia rusticana*) as food and medicine from antiquity to the present: Emphasis on the nordic countries. J Herbs, Spices Med Plants. 2011; 17 (3): 197–213.
6. Wedelsbäck Bladh K. Biodiversity in Nordic Horseradish. Swedish University of Agricultural Sciences; 2014.
7. Ciska E, Horbowicz M, Rogowska M, Kosson R, Drabińska N, Honke J. Evaluation of Seasonal Variations in the Glucosinolate Content in Leaves and Roots of Four European Horseradish (*Armoracia rusticana*) Landraces. Polish J Food Nutr Sci. 2017; 67 (4).
8. Singh A. Glucosinolates and Plant Defense. In: Mérillon J-M, Ramawat KG, editors. Glucosinolates. Cham: Springer International Publishing; 2017. p. 237–46.
9. Agneta R, Rivelli AR, Ventrella E, Lelario F, Sarli G, Bufo SA. Investigation of glucosinolate profile and qualitative aspects in sprouts and roots of horseradish (*Armoracia rusticana*) using LC-ESI-hybrid linear ion trap with fourier transform ion cyclotron resonance mass spectrometry and infrared multiphoton dissocia. J Agric Food Chem. 2012; 60 (30): 7474–82.
10. Agneta R, Lelario F, De Maria S, Möllers C, Bufo SA, Rivelli AR. Glucosinolate

profile and distribution among plant tissues and phenological stages of field-grown horseradish. Phytochemistry. 2014; 106: 178–87.

11. Bones AM, Rossiter JT. The myrosinase-glucosinolate system, its organization and biochemistry. Physiol Plant. 1996; 97: 194–208.

12. Kim MG, Lee HS. Growth-inhibiting activities of phenethyl isothiocyanate and its derivatives against intestinal bacteria. J Food Sci. 2009; 74 (8): 467–71.

13. Kaiser SJ, Mutters NT, Blessing B, Günther F. Natural isothiocyanates express antimicrobial activity against developing and mature biofilms of *Pseudomonas aeruginosa*. Fitoterapia. 2017; 119: 57–63.

14. Dias C, Aires A, Saavedra MJ. Antimicrobial activity of isothiocyanates from cruciferous plants against methicillin-resistant *Staphylococcus aureus* (MRSA). Int J Mol Sci. 2014; 15 (11): 19552–61.

15. Chacon PA, Buffo RA, Holley RA. Inhibitory effects of microencapsulated allyl isothiocyanate (AIT) against *Escherichia coli* O157:H7 in refrigerated, nitrogen packed, finely chopped beef. Int J Food Microbiol. 2006; 107 (3): 231–7.

16. Delaquis PJ, Sholberg PL. Antimicrobial Activity of Gaseous Allyl Isothiocyanate. J Food Prot. 1997; 60 (8): 943–7.

17. Mari M, Leoni O, Iori R, Cembali T. Antifungal vapour-phase activity of allyl-isothiocyanate against Penicillium expansum on pears. Plant Pathol. 2002; 51 (2): 231–6.

18. Lopes LF, Bordin K, de Lara GH, Saladino F, Quiles JM, Meca G, et al. Fumigation of Brazil nuts with allyl isothiocyanate to inhibit the growth of *Aspergillus parasiticus* and aflatoxin production. J Sci Food Agric. 2017 Jul 4; 28 (3): 303–25.

19. Wu H, Zhang GA, Zeng S, Lin KC. Extraction of allyl isothiocyanate from horseradish (*Armoracia rusticana*) and its fumigant insecticidal activity on four stored-product pests of paddy. Pest Manag Sci. 2009; 65 (9): 1003–8.

20. Santos JC, Faroni LRA, Sousa AH, Guedes RNC. Fumigant toxicity of allyl isothiocyanate to populations of the red flour beetle Tribolium castaneum. J Stored Prod Res. 2011; 47 (3): 238–43.

21. Ioannides C, Konsue N. A principal mechanism for the cancer chemopreventive activity of phenethyl isothiocyanate is modulation of carcinogen metabolism. Drug Metab Rev. 2015; 47 (3): 356–73.

22. DeVore NM, Smith BD, Wang JL, Lushington GH, Scott EE. Key residues controlling binding of diverse ligands to human cytochrome P450 2A enzymes. Drug Metab Dispos. 2009; 37 (6): 1319–27.

23. Konsue N, Ioannides C. Tissue differences in the modulation of rat cytochromes P450 and phase II conjugation systems by dietary doses of phenethyl isothiocyanate. Food Chem Toxicol. 2008; 46 (12): 3677–83.

24. Hangen L, Bennink MR. Consumption of Black Beans and Navy Beans (*Phaseolus vulgaris*) Reduced Azoxymethane-Induced Colon Cancer in Rats Consumption of Black Beans and Navy Beans (*Phaseolus vulgaris*) Reduced Azoxymethane-Induced Colon Cancer in Rats. Nutr Cancer. 2009; 44 (1): 60–5.

25. Yu C, Gong AY, Chen D, Solelo Leon D, Young CY, Chen XM. Phenethyl isothiocyanate inhibits androgen receptor-regulated transcriptional activity in prostate cancer cells through suppressing PCAF. Mol Nutr Food Res. 2013 ;57 (10): 1825–33.

26. Multhoff G, Molls M, Radons J. Chronic inflammation in cancer development. Front Immunol. 2012; 2: 1–17.

27. Wagner AE, Boesch-Saadatmandi C, Dose J, Schultheiss G, Rimbach G. Anti-inflammatory potential of allyl-isothiocyanate - role of Nrf2, NF-κB and microRNA-155. J Cell Mol Med. 2012; 16 (4): 836–43.

28. Thejass P, Kuttan G. Allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC) inhibit tumour-specific angiogenesis by downregulating nitric oxide (NO) and tumour necrosis factor-α (TNF-α) production. Nitric Oxide - Biol Chem. 2007; 16 (2): 247–57.

29. Gong A, He M, Vanaja DK, Yin P, Jeffrey Karnes R, Young CYF. Phenethyl isothiocyanate inhibits STAT3 activation in prostate cancer cells. Mol Nutr Food Res. 2009; 53 (7): 878–86.

30. Subedi L, Venkatesan R, Kim SY. Neuroprotective and anti-inflammatory activities of allyl isothiocyanate through attenuation of JNK/NF-κB/TNF-α

signaling. Int J Mol Sci. 2017;18(7):1–16.

31. Liu Y, Dey M. Dietary Phenethyl Isothiocyanate Protects Mice from Colitis Associated Colon Cancer. Int J Mol Sci. 2017; 18 (9): 1908.

32. Xiao D, Srivastava SK, Lew KL, Zeng Y, Hershberger P, Johnson CS, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. Carcinogenesis. 2003; 24 (5): 891–7.

33. Geng F, Tang L, Li Y, Yang L, Choi KS, Kazim AL, et al. Allyl isothiocyanate arrests cancer cells in mitosis, and mitotic arrest in turn leads to apoptosis via Bcl-2 protein phosphorylation. J Biol Chem. 2011; 286 (37): 32259–67.

34. Øverby A, Zhao CM, Bones AM, Chen D. Naturally occurring phenethyl isothiocyanate-induced inhibition of gastric cancer cell growth by disruption of microtubules. J Gastroenterol Hepatol. 2014; 29 (S4): 99–106.

35. Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, et al. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. Cancer Cell. 2006; 10 (3): 241–52.

36. Wang J, Luo B, Li X, Lu W, Yang J, Hu Y, et al. Inhibition of cancer growth in vitro and in vivo by a novel ROS-modulating agent with ability to eliminate stem-like cancer cells. Cell Death Dis. 2017; 8 (6): 1-9.

37. Chen G, Chen Z, Hu Y, Huang P. Inhibition of Mitochondrial Respiration and Rapid Depletion of Mitochondrial Glutathione by β-Phenethyl Isothiocyanate: Mechanisms for Anti-Leukemia Activity. Antioxid Redox Signal. 2011 ;15 (12): 2911–21.

38. Xiao D, Powolny AA, Moura MB, Kelley EE, Bommareddy A, Kim SH, et al. Phenethyl isothiocyanate inhibits oxidative phosphorylation to trigger reactive oxygen species-mediated death of human prostate cancer cells. J Biol Chem. 2010; 285 (34): 26558–69.

39. Thomson SJ, Brown KK, Pullar JM, Hampton MB. Phenethyl isothiocyanate triggers apoptosis in jurkat cells made resistant by the overexpression of Bcl-2. Cancer Res. 2006; 66 (13): 6772–7.

40. Gao N, Budhraja A, Cheng S, Liu E-H, Chen J, Yang Z, et al. Phenethyl

isothiocyanate exhibits antileukemic activity in vitro and in vivo by inactivation of Akt and activation of JNK pathways. Cell Death Dis. 2011;2(4): 1-9.

41. Cuddihy SL, Brown KK, Thomson SJ, Hampton MB. Induction of apoptosis by phenethyl isothiocyanate in cells overexpressing Bcl-XL. Cancer Lett. 2008; 271 (2): 215–21.

42. Chen YR, Han J, Kori R, Tony Kong AN, Tan TH. Phenylethyl isothiocyanate induces apoptotic signaling via suppressing phosphatase activity against c-Jun N-terminal kinase. J Biol Chem. 2002; 277 (42): 39334–42.

43. Satyan KS, Swamy N, Dizon DS, Singh R, Granai CO, Brard L. Phenethyl isothiocyanate (PEITC) inhibits growth of ovarian cancer cells by inducing apoptosis: Role of caspase and MAPK activation. Gynecol Oncol. 2006; 103 (1): 261–70.

44. Tusskorn O, Prawan A, Senggunprai L, Kukongviriyapan U, Kukongviriyapan V. Phenethyl isothiocyanate induces apoptosis of cholangiocarcinoma cells through interruption of glutathione and mitochondrial pathway. Naunyn Schmiedebergs Arch Pharmacol. 2013; 386 (11): 1009–16.

45. Cavell BE, Alwi SSS, Donlevy AM, Proud CG, Packham G. Natural product-derived antitumor compound phenethyl isothiocyanate inhibits mTORC1 activity via TSC2. J Nat Prod. 2012; 75 (6): 1051–7.

46. Jeong Y, Cho H, Chung F, Wang X. Isothiocyanates suppress the invasion and metastasis of tumors by targeting FAK / MMP-9 activity. Oncotarget. 2017; 8 (38): 63949–62.

47. Wu X, Zhu Y, Yan H, Liu B, Li Y, Zhou Q, et al. Isothiocyanates induce oxidative stress and suppress the metastasis potential of human non-small cell lung cancer cells. BMC Cancer. 2010; 10: 269.

48. Kawakishi S, Namiki M. Decomposition of Allyl Isothiocyanate in Aqueous Solution. Agric Biol Chem. 1969; 33 (3): 452–9.

49. Ohta Y, Takatani K, Kawakishi S. Decomposition Rate of Allyl Isothiocyanate in Aqueous Solution. Biosci Biotechnol Biochem. 1995; 59 (1): 102–3.

50. Hrabcova H, Velíšek J, Pecháček R. Decomposition Products of Allyl Isothiocyanate in Aqueous. J Agric Food Chem. 1997; 45 (12): 4584–8.

51. Chen C, Ho C. Thermal Degradation of Allyl Isothiocyanate in Aqueous Solution. J Agric Food Chem. 1998; 46 (1): 220–3.

52. Chen C, Rosen RT, Ho C. Analysis of Thermal Degradation Products of Allyl Isothiocyanate and Phenethyl Isothiocyanate. Flavor Analysis. American Chemical Society; 1998. 152-166 p.

53. Neoh TL, Yamamoto C, Ikefuji S, Furuta T, Yoshii H. Heat stability of allyl isothiocyanate and phenyl isothiocyanate complexed with randomly methylated β-cyclodextrin. Food Chem. 2012; 131 (4): 1123–31.

54. Plackett D, Ghanbari-Siakali A, Szente L. Behavior of a- and b-Cyclodextrin-Encapsulated Allyl Isothiocyanate as Slow-Release Additives in Polylactide-co-Polycaprolactone Films. Polym Polym Compos. 2007; 105: 2850–7.

55. Li Y, Teng Z, Chen P, Song Y, Luo Y, Wang Q. Enhancement of aqueous stability of allyl isothiocyanate using nanoemulsions prepared by an emulsion inversion point method. J Colloid Interface Sci. 2015; 438: 130–7.

56. Trivellini A, Lucchesini M, Maggini R, Mosadegh H, Villamarin TSS, Vernieri P, et al. Lamiaceae phenols as multifaceted compounds: Bioactivity, industrial prospects and role of "positive-stress." Ind Crops Prod. 2016; 83: 241–54.

57. Dorman HJD, Bachmayer O, Kosar M, Hiltunen R. Antioxidant Properties of Aqueous Extracts from Selected Lamiaceae Species Grown in Turkey. J Agric Food Chem. 2004;52 (4): 762–70.

58. Shan B, Cai YZ, Sun M, Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J Agric Food Chem. 2005; 53 (20): 7749–59.

59. Zheng W, Yang SY. Antioxidant Activity and Phenolic Compounds in Selected Herbs. J Agric Food Chem. 2001; (49): 5165–70.

60. Cai R, Httiarachchy NS. High-Performance Liquid Chromatography Determination of Phenolic Constituents in 17 Varieties of Cowpeas. J Agric Food Chem. 2003; 51 (6): 1623–7.

61. Monschein M, Jaindl K, Buzimkić S, Bucar F. Content of phenolic compounds in wild populations of Epilobium angustifolium growing at different altitudes. Pharm Biol. 2015; 53 (11): 1576–82.

62. Rieger G, Müller M, Guttenberger H, Bucar F. Influence of altitudinal variation on the content of phenolic compounds in wild populations of *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*. J Agric Food Chem. 2008; 56 (19): 9080–6.

63. Schneider I. Arzneipflanzen mit inhibitorischer Wirkung auf den Lipoxygenase katalysierten Arachidonsäuremetabolismus. Faculty of Natural Sciences, University of Graz; 2003.

64. Wilson EA, Bindler F, Marchioni E, Bergaentzlé M, Benrabah T, Ennahar S. SPE for the simultaneous determination of various isothiocyanates. J Sep Sci. 2012; 35 (23): 3369–74.

65. Tomsone L, Kruma Z, Galoburda R, Talou T. Composition of Volatile Compounds of Horseradish Roots (*Armoracia rusticana* L.) Depending on the Genotype. Proc Latv Univ Agric. 2013; 29 (1): 1–10.

66. Jiang ZT, Li R, Yu JC. Pungent components from thioglucosides in *Armoracia rusticana* grown in China, obtained by enzymatic hydrolysis. Food Technol Biotechnol. 2006; 44 (1): 41–5.

6. Appendix

6.1. Calibration Curves