
# Study On Horseradish (*Armoracia Rusticana*) Essential Oil And Comparison With The Related Species – Debreceni Horseradish (*Armoracia Macrocarpa*).

Doktori (PhD) értekezés

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Nyilatkozatok

*Ezen értekezést a Debreceni Egyetem Természettudományi Doktori Tanács Juhász-Nagy Pál Doktori Iskola Biológia Programja keretében készítettem a Debreceni Egyetem természettudományi doktori (Ph.D) fokozatának elnyerése céljából.*

*Debrecen, 2016.10.17*

……..

*Nguyen Minh Nhat*

*Tanusítom, hogy* ***Nguyen Minh Nhat*** *doktorjelölt 20011-2016 között a fent megnevezett Doktori Iskola biológia programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.*

*Debrecen, 2016.10.17*

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*Dr. Vasas Gábor*

**Study On Horseradish (*Armoracia Rusticana*) Essential Oil And Comparison With The Related Species – Debreceni Horseradish (*Armoracia Macrocarpa*).**

Értekezés a doktori (Ph.D) fokozat megszerzése érdekében
a biológia tudományágban
Írta: Nguyen Minh Nhat, pharmacist
Készült a Debreceni Egyetem Juhász-Nagy Pál Doktori Iskola (biológia doktori programja) keretében
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## Introduction

*Armoracia rusticana G. Gaertn., B. Mey. & Scherb.* (commonly known as horseradish) is well known for the irritating, pungent smell and bitter taste. In Hungary, horseradish has been cultivated and used extensively in food industry, as well as in traditional medicine. Characteristic smell, taste and possible pharmacological effects comes from the plant' s essential oil. Developing essential oil extracting technology is not only proved to be useful for economical purpose, but also for scientific research due to the high content of isothiocyanate, a potential anti-carcinogenic agent present in the essential oil. Therefore, this study starts with the development of oil extracting technology, first in laboratory-scaled and then medium-scaled distillation. The efficiency of the new technology is taken under investigation by examining the yield of extracted oil, the completion of extraction process and the quality (content) of essential oil.

Although there have been studies on horseradish's isothiocyanates content, at the moment of this study, there has been no analytical investigation on neither isothiocyanate, glucosinolate profile nor the enzymatic activities in horseradish cultivated in Hungary. The analytical study starts with gas chromatography and mass spectrometry analysis on horseradish essential oil in order to reveal the full profile of horseradish isothiocyanates, which was compared to data collected from literature after for checking the quality of the extracted oil. The second part of the analytical study concentrates on development of the novel capillary electrophoresis method for instantly separation and detection of isothiocyanates and their parent molecules (glucosinolates) as well as revealing activity of myrosinase enzyme on the conversion of glucosinolates into

isothiocyanate.

*Armoracia macrocarpa (Waldst. & Kit.) Baumg.* or Debreceni horseradish, is the relative species of *Armoracia rusticana*. Debreceni horseradish has been used for condiment purpose and known as "sweet radish". Unlike its famous relative, there is neither information on *Armoracia macrocarpa*' s phytochemistry, anatomical structure nor enzymatic activities. The final part of this study gives the comparison on glucosinolate contents (by liquid chromatography and mass spectrometry), anatomical structure (by cross sections) and enzymatic activities (by gel electrophoresis and spectrophotometry) between *Armoracia rusticana* and *Armoracia macrocarpa*.

# Chapter I

## Literature Review

### 1. THE PLANT

*Armoracia rusticana*, *Cochlearia armoracia*, and *Armoracia lapathifolia* are scientific names that refer to a perennial plant of Mustard family (genera-group *Cardamineae* of *Brassicaceae*) commonly known as horseradish (Mohlenbrock 1980).The plant can reach the height of 120cm. It has a hardy glabrous stem, from which wavy margin leaves arise directly (cauline leaf) following a circular arrangement pattern (basal rosette). Horseradish leaf is described to have a length of 30–100cm, a cordate base, long petiole, and the shape slightly varying from the lower to the uppermost leaf. Whereas a shorter petiole and a lobe shape with entire or serrate margin are characteristics of lower leaves, upper leaves have a narrow base, obtuse apex, oblong or lanceolate shape with crenate or serrate margin. The margin is linear or almost entire in the case of uppermost leaves (Mohlenbrock 1980).Horseradish has white, tetramerous flowers arranged in racemes and a smooth, brown angustiseptate fruit—a fruit flattened at a right angle to the septum, which usually contains very few (≤ 6) or no seeds. In addition, the lack of evidence that horseradish grows from seeds suggests sterility (Sampliner and Miller 2009).

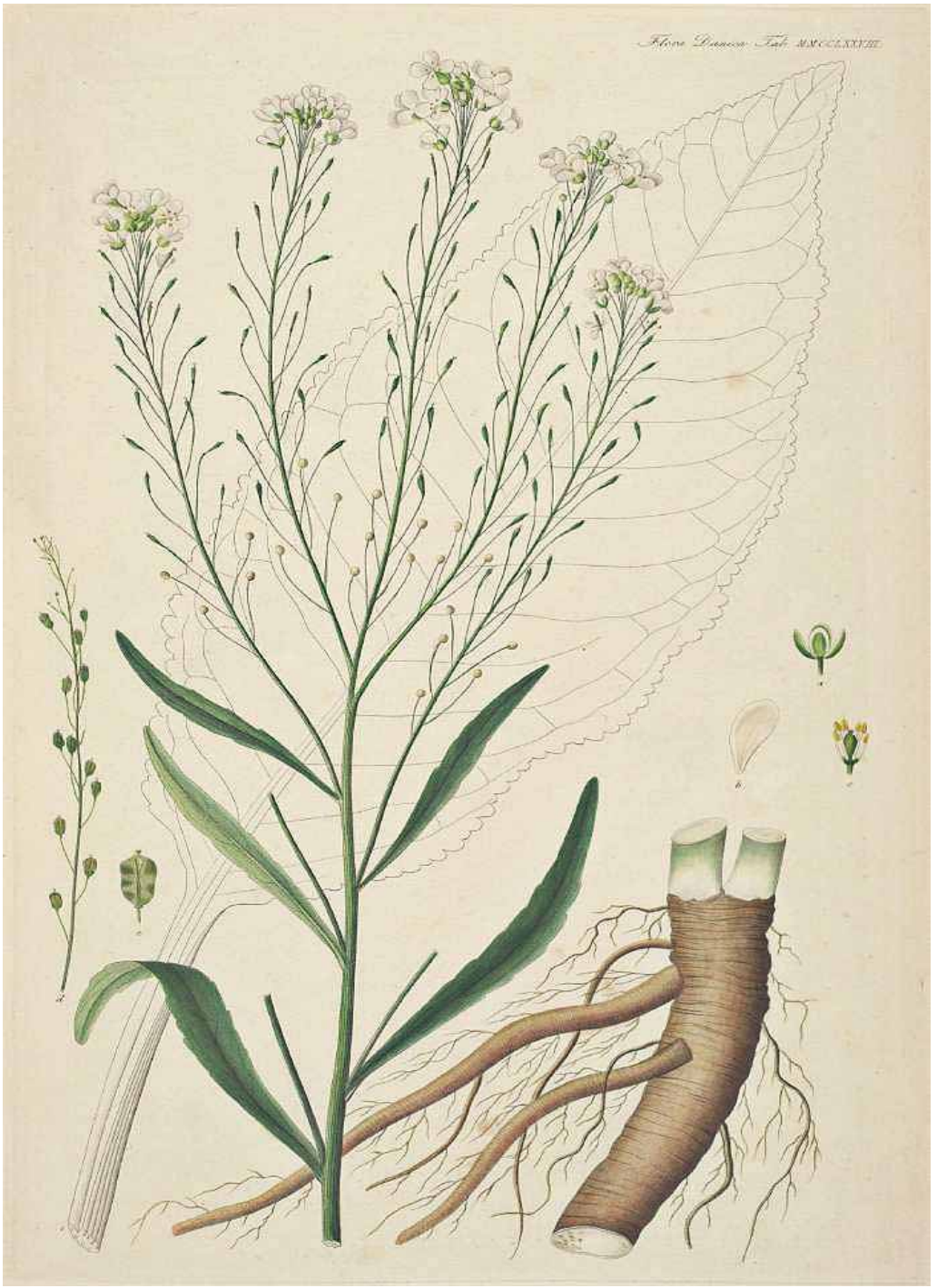


**Figure 1.** *Armoracia rusticana. Adapted from http://plantillustrations.org.*

## 2. THE ORIGIN

Horseradish is believed to be native to Eastern European countries (such as Romania or Ukraine) even though it can now be found throughout Europe. Horseradish occurrence was found only associated in areas where there are people. No information about the wild population of the plant has been found. It is suggested that the wild population of horseradish may have become extinct or it may have been derived from related species such as *Armoracia macrocarpa* and *Armoracia sisymbroides (DC.) N.Busch ex Ganesh*. Its ability to spread by rhizome could mean that horseradish is a problematic weed, though it is also possible that its wild population is just yet to be found (Sampliner and Miller 2009).

## 3. THE RELATIVE SPECIES

*Armoracia macrocarpa* is native to Eastern Europe, specifically to the marshes of the Central Danube Basin (e.g., in Hungary, Czech Republic, Romania, and Bulgaria), whereas Siberia is the native land of *Armoracia sisymbroides*.

*Armoracia macrocarpa* grows in reeds (*Scirpo-Phragmitetum austro-orientale*), wet meadows (*Phalaridetum arundinaceae*) and salt meadows (*Agrosti (o)-Alopecuretum pratensis*). It favors alkaline soils, salt tolerant, wet or water covered adherent loam, clay, salt soils, rich in nutrients and alkaline minerals. In Hungary, *Armoracia macrocarpa* can be found in the North Hungarian Mountains (Mátra margins), Great Hungarian Plain (Danube region, Danube-Tisza köze region and Tiszántúl region) and South Transdanubia. It has a fragmented distribution, especially in northeastern parts of the country. Due to

its habitat specialization and a continuing decline in extent and quality of its habitat, *Armoracia macrocarpa* is classified as rare (Hungary, Romania) or very rare (Serbia). *A. macrocarpa* and *A. rusticana* are nearly identical. The flowers and fruits of *A. macrocarpa* are larger than those of *A. rusticana*; further, *A. macrocarpa* fruits contain numerous seeds, while the fruits of *A. rusticana* rarely contain seeds. *Armoracia macrocarpa* seems to have much larger inflorescences than *A. rusticana* .(Sampliner and Miller 2009).

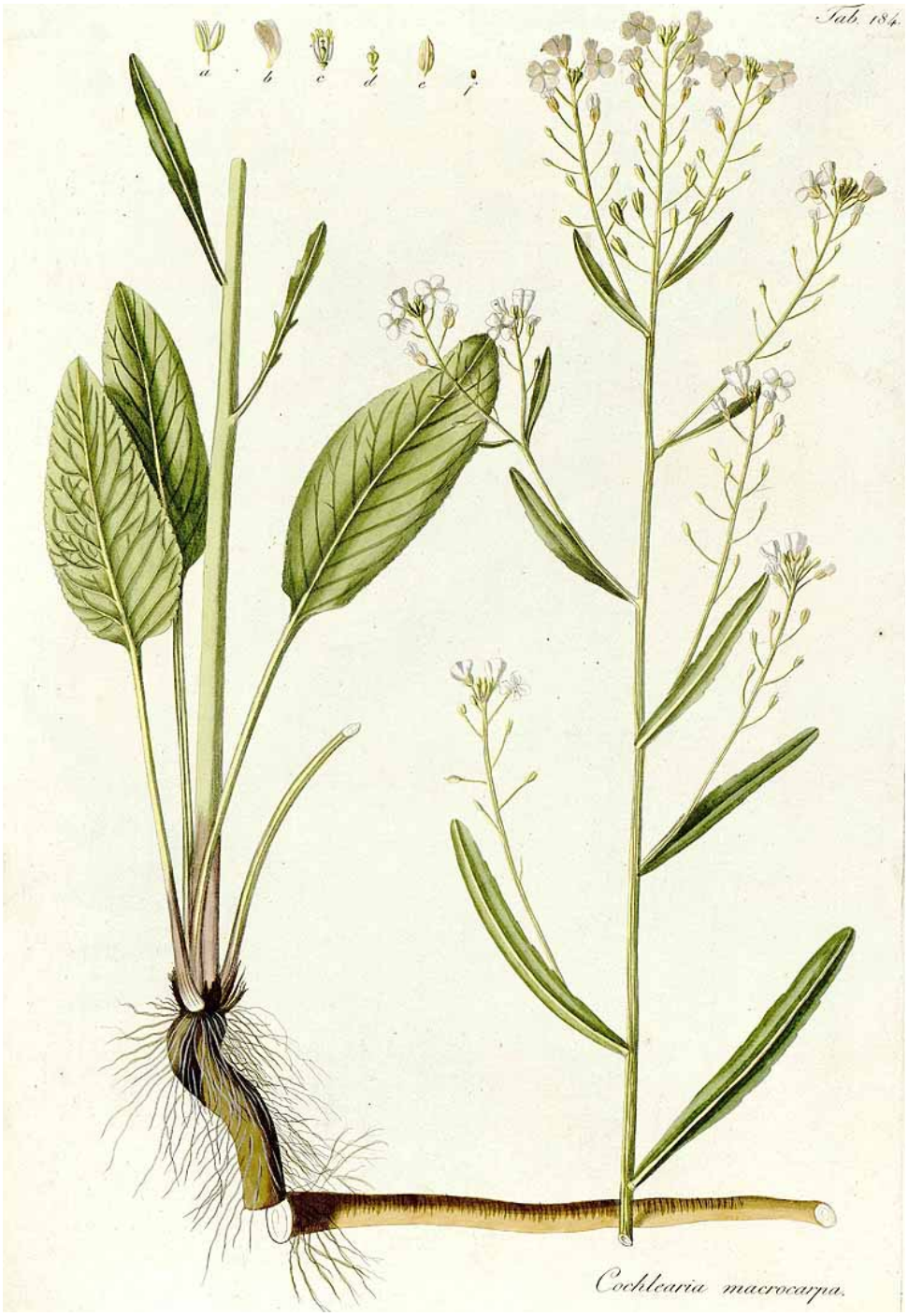



**Figure 2.** *Armoracia macrocarpa. Adapted from http://plantillustrations.org.*

*Armorcia sisymbrioides* is distinguishable from other relative species by its whitish, glaucous leaves. Moreover, its cauline leaves are auriculate. Neither of *Armoracia rusticana* and *A. macrocarpa* have these features (Sampliner and Miller 2009). There is little or no information on these two species of *Armoracia* genus, nor on the relation between these species and horseradish. Research in this field could help improve current horseradish crops, via traditional breeding or biotechnological techniques.

## 4. THE CROP

For propagation, the roots are used exclusively. Horseradish root is white, cylindrical or tapering, which can reach a length of 60 cm in loose soil and consist of several lateral roots. Deep, silty loam soils with good drainage and temperate climates are favorable conditions for both annual and perennial horseradish crops. For commercial production, horseradish is usually cultivated as an annual crop, which usually starts with plantation in early spring because the roots need the higher temperatures of the summer (15–27$^{o}$C) and the lower temperatures of the end of summer and fall (11–22$^{o}$C) to develop. Usually the whole root is harvested. In the case of perennial production, the underground shoot grown from original root is collected, and the original root is left in the field for regeneration. Major horseradish-growing countries are the United States and Hungary (Shehata et al. 2009).

## 5. POSSIBLE USES

Due to the characteristic pungent, intensive lachrymatory odor and taste of the

root, horseradish is cultivated mostly for condiment production. It is also used as a type of food and for traditional medical purposes. For example, horseradish has been used to ease pain such as low back pain and pain associated with sciatica and rheumatism. It is also traditionally used as a urinary, gastrointestinal, and respiratory aid, for toothache, and as aphrodisiac. Combination of horseradish root and honey in warm water is made for treating influenza. However, the underlying mechanism of possible medicinal benefits of horseradish remains unclear (Sampliner and Miller 2009).

## 6. POSSIBLE PHARMACOLOGICAL STUDIES

Isothiocyanates (ITCs) are the products of hydrolysis reaction of glucosinolates (GLSs), which occurs when the plant tissues are damages. ITCs are potential anti-carcinogenic agents that not only inhibit the development of cancer cells but also eliminate established cancer cells (Zhang 2004). The mechanism underlying anti-carcinogenic effect of ITC is unclear even though there have been many suggested hypotheses based on observations in animal and human cell studies. There are yet no clinical trials on ITCs or their precursors, GLSs (Valgimigli and Iori 2009).

### 6.1. ITCs inhibit carcinogen-activating enzymes and induce carcinogen-detoxifying enzymes.

The underlying pharmacology mechanisms of ITCs on cancer cells could be due to the possibilities of inhibiting carcinogen-activating enzymes and inducing carcinogen-detoxifying enzymes. Cellular enzymes such as those belonging to the cytochrome P450 (CYP) family are known as carcinogen-

activating enzymes due to their ability to transform pro-carcinogens into carcinogens, the active form that can be harmful to cells. ITCs showed the ability to inhibit or down-regulate these enzymes. In a study where animals were treated with carcinogenic agent (nitrosamine), ITCs, especially arylalkyl ITCs such as PEITC, inhibited CYP enzymes that are necessary for nitrosamine activation (Hecht 2000).

Phase II enzymes such as quinone reductase 1 (QR1), glutathione S-transferase (GST), and heme oxygensase 1 (HO-1) play important roles in cellular defense mechanism against oxidants and carcinogens and are known as carcinogen-detoxifying enzymes. Inducing gene transcriptions of those enzymes is thought to be one of the anti-carcinogenic strategies used by ITC. Modulation of phase II enzymes is the consequence of the interaction between ITCs and the complex consisting of a nuclear transcription factor, Nrf2 (NF-E2–related factor 2), and a protein anchored to the actin cytoskeleton known as Keap1 (Kelch-like ECH-associated [erythroid cell-derived protein with CNC homology] protein 1). Binding of ITC to the complex involves the reaction of ITC and protein sulfhydryl residues of Keap1, leading to the dissociation of the complex into Nrf2 and Keap1, as it is observed that Nrf2 concentration is significantly increased when treating human hepatoma HepG2 cells with 25 μM AITC (Jeong et al. 2005). Free Nrf2 then translocates into the nucleus where together with other transcription factors, it interacts with antioxidant response element (ARE), resulting in the activation of gene transcription for carcinogen-detoxifying enzymes (Dinkova-Kostova et al. 2002).

**6.2. ITCs trigger apoptosis pathways.**

Apoptosis or programmed cell death results from cleavage of specific cellular substrates caused by the activity of aspartate-specific cysteine protease known as caspase. Main signaling pathways leading to caspase activation are via death receptors such as tumor necrosis factor (TNF) receptors and via mitochondria. AITC (10 μM) activated caspase 9 (mitochondria pathway), caspase 8 (death receptor pathway), and caspase 12 (estrogen receptor [ER] pathway) in conjugation with caspase 3 activation in human leukemia HL60 cells (R. Yu et al. 1998).

Mitochondrial pathway is regulated largely by members of Bcl2 family—antiapoptotic members (Bcl-2, Bcl-x L), proapoptotic members (Bax, Bak, Bok), and BH3-only protein (Bid, Bad, Bin). Changes in the regulators of mitochondria pathway were accompanied with ITCs treatment in several studies. Apart from the effect on regulators, ITCs are suggested to exert direct effect on mitochondria itself, causing the release of cytochrome c. By binding to a heme group, cytochrome c becomes holocytochrome c, which has the ability to activate caspases (Srivastava et al. 2003; Singh et al. 2004; Xiao et al. 2003; Fimognari et al. 2002; Chen et al. 1998; Xu and Thornalley 2001).

### 6.3. ITCs inhibit cell cycle progression.

The key regulator molecules of cell proliferation through the cell cycle are cyclins – the regulatory proteins that activate a specific class of enzymes known as cyclin-dependent kinase (cdk). Together, they act as an activated complex that pushes the cell through certain stages of the cycle. Each stage of the cell cycle is marked with the activity of certain type of cyclin and kinases. AITC caused cell cycle arrest in the G1 phase and in the G2/M phase (Zhang, Tang,

and Gonzalez 2003; Tang and Zhang 2004; Smith et al. 2004). Cell cycle arrest after AITC treatment was accompanied with down-regulation of cyclin B, cdk1, cdc25B, cdc25C, and tubulin disruption, suggesting that the inhibitory effect of ITCs is a complex process. Cell types, dose and time of exposure may influence the inhibitory effect of ITCs (Clarke, Dashwood, and Ho 2008; Hwang and Lee 2006; Chiao et al. 2002).

### 6.4. ITCs inhibit pro-inflammatory and pro-carcinogen signaling factor.

Cancer can be the consequence of chronic inflammation process in which pro-inflammatory and pro-carcinogen signaling factors secreted by cells play important roles. These factors are cell-derived mediators (e.g., nitric oxide (NO), prostaglandin E2 (PGE2), and tumor necrosis factor (TNF)). Inhibiting the production or secretion of those molecules is possibly one of the anticarcinogenic mechanisms by which ITCs act (Gerhäuser et al. 2003; Ippoushi et al. 2002).

### 6.5. ITCs possess antimicrobial and antioxidant activities.

ITCs possess antibacterial effect against several bacteria, which is related to anticarcinogenic effects, e.g., against *Helicobater pylori*, one possible cause of stomach cancer (Zsolnai 1971). Bactericidal effect against *Helicobacter pylori*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus mutans*, *Penicillium notatum*, *Bacillus cereus*, and *Vibrio parahaemolyticus* was reported at AITC concentration of 3.8–16.7 μM, with an activity that was 7.8–20.5 times less than that of PEITC (Shin, Masuda, and Naohide 2004; Luciano and Holley 2009; Tunc et al. 2007). There are several

suggested possibilities linked to the antibacterial activity of ITCs, e.g., by breaking down enzyme S-S bridges, ITCs can inactivate several pathogen enzymes; by the uncoupling action of oxidative phosphorylation in mitochondria, leading to the obstruction of adenosine triphosphate (ATP) synthesis in bacterial cell…. Apart from antibacterial effect, ITCs also possess antiprotozoal and antifungal activities (Xiao et al. 2003), whose action mechanisms are also under investigation. AITC has fungicidal activity against *Aspergillus flavus, Endomyces fibuliger, Penicillium commune, Penicillium corylophilum, Penicillium discolor, Penicillium palitans, Penicillium polonicum, Penicillium raqueforti, Penicillium solitum, and Pichia anomala* (Nielsen and Rios 2000).

**6.6. ITCs are considered to be oxidants themselves.**

The doses of ITCs needed to cause these effects are in much higher amount (i.e. at least 200–400 μg/kg body weight in animal studies to show clear oxidant behavior) than the amount that humans are normally exposed to, with an average estimate of less than 1mg/day (10 μg/kg body weight) in case of AITC (Zhang, Tang, and Gonzalez 2003). The effect is possibly resulted from the formation of super-oxide when –N=C=S group of AITC reacts with Cu(I) and hydrogen peroxide; decreasing intracellular thiol pool because of conjugation reaction with GSH occurs after ITCs diffuse into the cell; induction of certain CYP enzymes that can transform procarcinogen to active carcinogen; rapid loss of transmembrane potential, mitochondrial damage, or loss of cytochrome c (Murata et al. 2000; Bruno and Njar 2007; Tang and Zhang 2005).

# Chapter II

## Essential Oil Distillation Technology

*"Distillation of aromatic plants simply implies vaporizing or liberating the oils from the plant cellular membranes in the presence of moisture, by applying high temperature and then cooling the vapor mixture to separate the oil from the water on the basis of the immiscibility and density of the essential oil with respect to water."*("Extraction Technologies for Medicinal and Aromatic Plants" 2016)

### 1. SMALL SCALED DISTILLATION

### 1.1. Materials and sample preparation

Fresh horseradish main and lateral roots were provided by KELET PRODUCT Zrt, Hungary. The fresh roots were used, otherwise, they were stored at 4°C for later studying. As the roots do not yield essential oil easily in their natural state and isothiocyanate is only produced when hydrolysis reaction is triggered (i.e. when the roots are damaged), horseradish roots (233 ± 38.74 g, 151.67 ± 1.52 g for main and lateral roots, respectively) were cut and grind in order to achieve paste-like condition prior to distillation by commercial electric grinder (BOSCH, CNCM13STI) for 5 minutes. The reaction of glucosinolate and myrosinase happens instantly upon the damage on the root due to the occurrence of the characteristic smell and lachrymatory effect. Root-paste was transferred immediately into a round flask to avoid losing volatile components, following by the addition of water. When the distillation was completed, the extracted essential oil was separated from watery extract by centrifugation at

13000 rpm for 5 minutes (Biofüge pico, Heraeus).

### 1.2. Methods

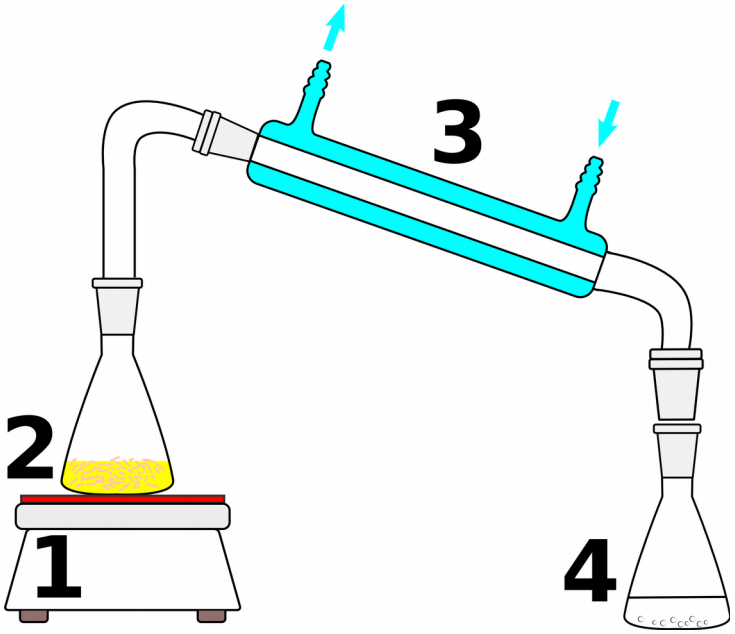



**Figure 3.** Horseradish essential oil extraction using hydro-distillation with direct heating mode. Diagram was made by INKSCAPE, version 0.91. Legends – 1: heating source, 2: plant materials in containing flask, 3: condenser, 4: oil collecting flask.

Water distillation (or hydro-distillation) is the simplest and oldest process available for obtaining essential oils from plants. The root-paste is almost entirely covered with water in the round flask, which is directly placed on an electric heater (ETA, 2017-202, level 3, approximately 120°C). As the water boils, the steam carries essential oil up to the condenser (figure 3). Dense materials from the sample tend to agglomerate on the bottom of the flask, therefore the water content must be enough to last throughout the process to

prevent overheat and char. Because mixing of the plant material during distillation was practically difficult in this stage, testing different amount (ml) of added water (1:1, 1:2, 1:3 volume to material mass) and distillation duration are the more appropriate approaches.

Another approach is to replace the direct heating mode by indirect heating mode, i.e. using heating media such as water bath, sand, liquid paraffin and sun flower oil. The advantages of indirect heating mode are the ease of controlling the end temperature and the increase of heating surface as the flask was almost covered in the heating media (figure 4).

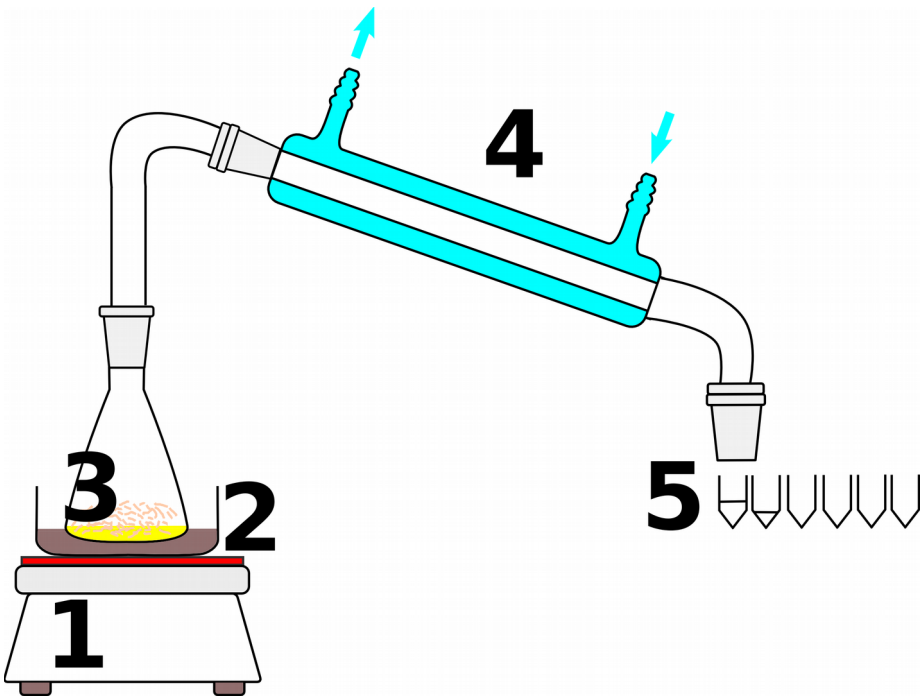



**Figure 4.** Horseradish essential oil extraction using hydro-distillation with water bath heating. Diagram was made by INKSCAPE, version 0.91. Legends – 1: heating source, 2: water bath, 3: plant materials containing flask, 4: condenser, 5: oil collecting tubes)

Attempt to totally avoid the direct contact between the materials and the flask wall, thus reducing the sample burning risk, is to exploit the heating mode using steam (figure 5). Water and plant materials are separated by introducing the perforated grid in the still. When the water level is kept below the grid, the essential oil is distilled by the rising steam from the boiling water.

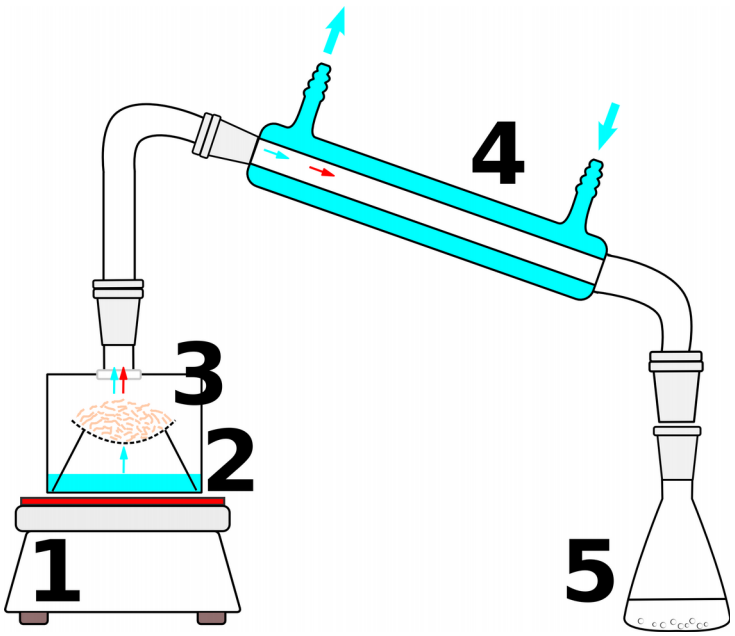



**Figure 5.** Horseradish essential oil extraction using steam distillation. Diagram was made by INKSCAPE, version 0.91. Legends – 1: heating source, 2: pressure cooker, 3: plant materials located on the grid, 4: condenser, 5: oil collecting tubes.

A fractional column was also introduced before the condenser to improve the process (fractional distillation) (figure 6). In this improved version, the temperature gradient is established along the column. When mixture is boiled,

its vapor travels and condenses at a certain section of the column where it will be “re-distillated” again. The same process occurs throughout the column. At the end, the components with lower boiling point will reach top of the column and travel to the condenser.

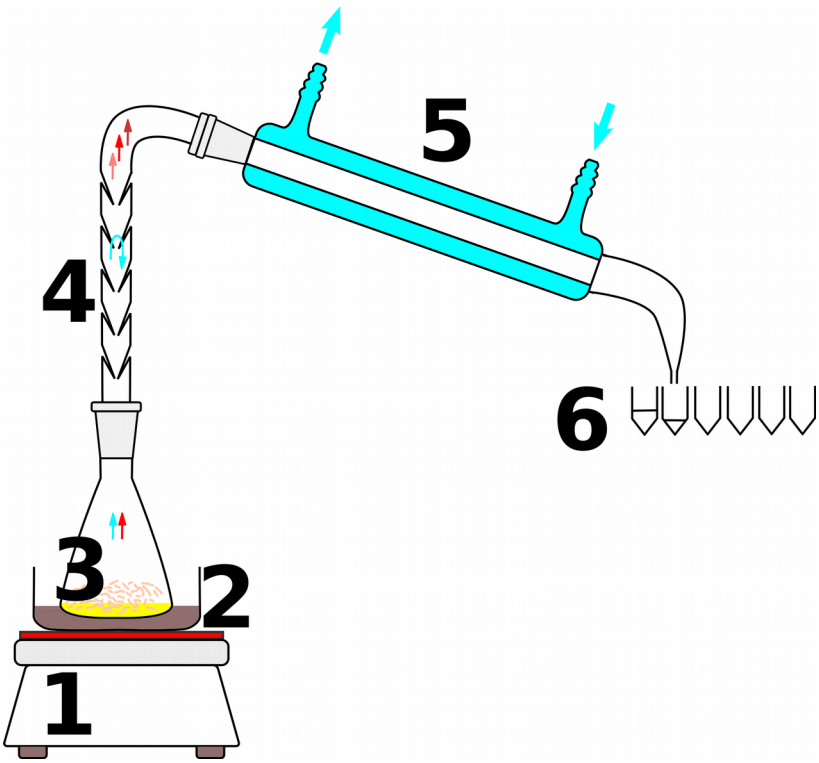



**Figure 6.** Horseradish essential oil extraction using fractional distillation. Diagram was made by INKSCAPE, version 0.91. Legends – 1: heating source, 2: water bath, 3: plant materials, 4: fractional column, 5: condenser, 6: oil collecting tubes.

### 1.3. Results and discussion

Direct contact of flask and electric heater is the serious drawback for distillation

using direct heating mode. Regardless of different amount of added water, the plant materials near the bottom of the still charred, affecting the yield, quality and odor of essential oil. Attempt to decrease heating power together with extend the distillation duration led to the decomposition of essential oil's components due to the prolonged interaction with hot water. Moreover, the insufficient rate of steam production due to the low heating power (<90°C) causes the reflux of oil back to the sample containing flask, leading to decomposition reaction and poor oil quality. The same phenomenon was observed in apparatus using steam heating, plus the contact surface area of the plant materials and steam is limited, i.e. plant material tended to aggregate, preventing steam from heating the upper, inner layers. In fractional distillation, longer distance and flow back of essential oil droplets are probably the explaination for discrepancy in essential oil amount.

In the improvised hydro-distillation using water bath heating, problems with burning and char of plant materials were solved. The flask was heated at constant temperature of boiling water (approximately 100°C). No material char was observed. The heating surface area was increased as the flask was almost entirely immersed into the boiling water. Produced steam was sufficient for the process. The yield of 0.071% (166.66 ± 27.54 µl) essential oil was extracted from fresh horseradish main roots (233 ± 38.74 g). For fresh lateral roots (151.67 ± 1.52 g), the yield of essential oil is 0.12% (187 ± 9.6 µl). The amount of water added following the ratio of 1:3 volume / material weight, results in the highest yield compared to other ratio in case of lateral root distillation. For the main roots distillation, less water was added (1:6 volume / material mass) because of their higher water content. The duration of 1.5 h was sufficient for complete extraction of essential oil.  In addition to provide the stable yield of

essential oil, this extracting method comes with simplicity and the ease of use, i.e. required minimum attention during the operation.

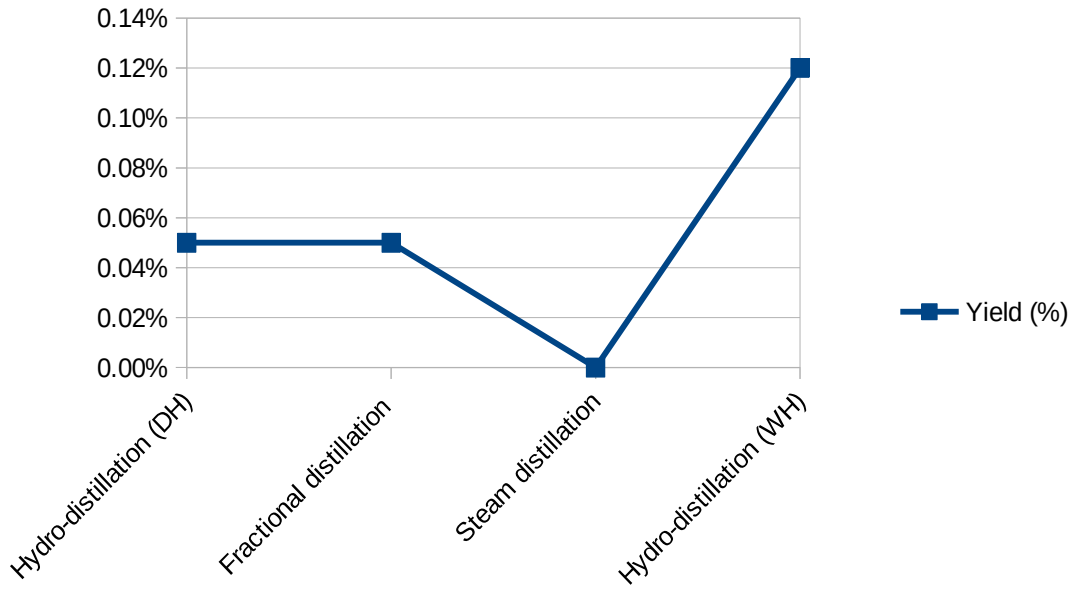



**Figure 7.** Comparing chart of horseradish essential oil yields from different distillation methods. Chart was made by LibreOffice Calc 5.2.1.

## 2. MEDIUM SCALED DISTILLATION

The medium-scaled distillation technology was built based on the experiences from the laboratory-scaled extracting methods. As the amount of plant material is much higher in this case (approximately 15 kg), different adjustments on influencing factors (e.g. duration of the process, amount of added water, heating temperature…) were crucial in order to reach the similar yield (0.07%) of laboratory-scaled experiments.

## 2.1 Materials and technology

The fresh fleshy horseradish roots were cultivated in Újléta and supplied by KELET PRODUCTION Zrt., Hungary. The plant materials (≈15.5 kg) were chopped, grind by commercial industrial grinder and transferred immediately into the still with great care due to the pungency of high material amount. The still and its components are made of stainless steel to avoid corrosiveness from essential oil. The plant material was mixed during distillation by a rotating frame with diagonal bars, which is introduced inside the still. Heating plates at the bottom (n=3) and heating rings on the wall (n=3) of the still ensure the sufficiency of heating surface area as well as heating power. The rotation (rpm) of mixing frame and power of heating plates as well as their activating order are accessible through a controlling panel located separately from the still. The spiral condenser was used to sufficiently process large amount of steam.

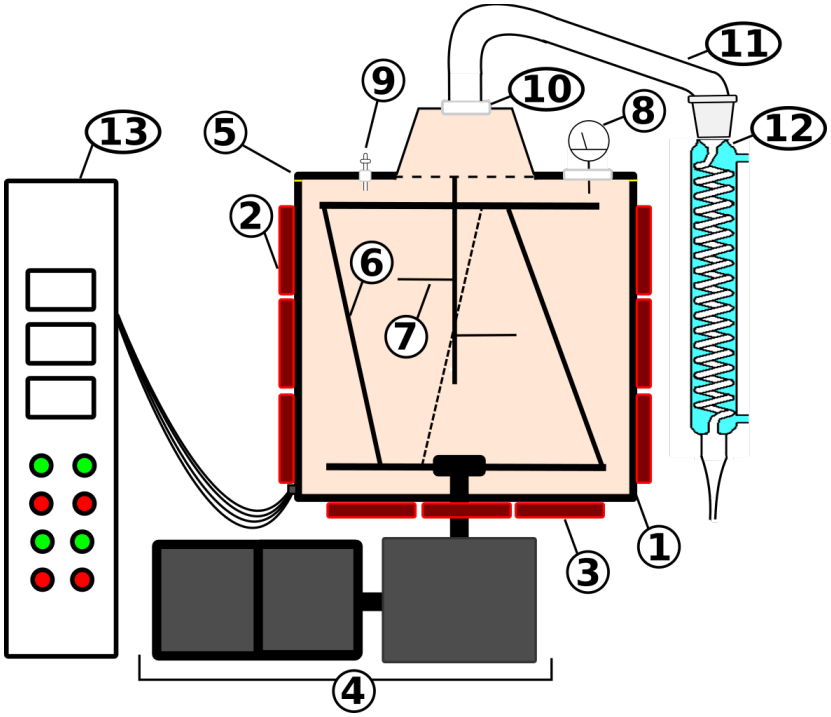



**Figure 8.** Diagram of mid-scaled distillation instrument. Diagram was made by INKSCAPE, version 0.91. Legends – 1: the tank, 2: heating rings, 3: heating plates, 4: rotating motor, 5: lid of the tank, 6,7: mixing frame with diagonal bars, 8: thermostat, 9: valve, 10: neck connector, 11: connecting tube, 12: condenser, 13: control panel.

The important tested factors of extracting technology includes: amount of water added to the system, optimum temperature and heating mode. Different amounts of added water (0, 250, 500, 750 mL) were tested and were combined with various heating program (90, 92.5, 95, 97.5$^{o}$C). Heating programs were designed based on the activating order of wall and bottom plates and the desired end-temperature of the system (90÷97.5$^{o}$C). The extracted essential oil was separated from the watery distillate by using massive centrifugation (Beckman Avanti J-25) at 13000 rpm for 10min.

## 2.2. Results and discussions

At the same heating program (95$^{o}$C), experiments with different added water amounts showed that slightly better yield (0.03% yield) of essential oil was achieved when no water was added compared to 0.02% essential oil yield in the other cases (i.e. 0.25, 0.5, 0.75 L). This can be explained based on the large amount of water comes from the fresh roots. It is important to note that adding more water into the system would result in high amount of watery extract. The heating program was designed as following: heating rings on the wall of the tank is activated in order for the temperature in the still to reach 70$^{o}$C (to decrease the heating duration of substance agglomerated at the bottom of the tank) and heating plates on the bottom is activated to reach desired end temperature. Low heating temperature (<95$^{o}$C) results in prolonged duration of the distillation while high temperature (>97$^{o}$C) more likely results in sample char. Good result was achieved when the setting temperature falls around 95$^{o}$C, i.e. better yield (0.05%) compared to other temperature settings (i.e. 90$^{o}$C – 0.02%, 92.5$^{o}$C – 0.03% and 97.5$^{o}$C – 0.03%). No sample burning was recorded but agglomerations of plant material were found at the bottom of the still by the end of the process, suggesting that the distillation may not reach it highest yield. However, this is an inevitable technical problem. The extracting process is considered to be completed in 2 h. Distillation of average 15 kg of fresh horseradish main roots using the optimal setting resulted in 12 mL essential oil (average yield 0.08%). On the other hands, distillation of fresh lateral roots (average yield of 0.01%) failed to achieve the expected yield regardless to different amount of added water. Various amounts oil were extracted from horseradish roots incubated in different period. In some cases, e.g., distillation

of roots stored for 21 days in cold room (4°C), better yield was achieved (i.e. 0.14%). However, due to the discrepancy of the results, different incubating periods and storing conditions are not a reliable method compared to the distillation using fresh roots. The newly developed extracting technology satisfied the requirements. It provides a stable yield of essential oil and similar to that from the laboratory-scaled experiments. It is easy to handling, operating and requires reasonable time of attention. The proposed technology is suitable for extracting high amount of essential oil from fresh horseradish roots. The investigation of the efficiency of the new method is discussed in the following chapter.

## Chapter III

## Analytical Studies

# 1. BACKGROUND INFORMATION

## 1.1. Glucosinolates (GLSs)

*A. rusticana* is rich in glucosinolates, the secondary products that play an important role in the plants defensive system. GLS is an organic anion that is stable and soluble in water (Oerlemans et al. 2006). GLS' s structure consists of β-thioglucoside *N*-hydroxysulfates with a side chain (R) and a sulfur-linked β-D-glucopyranose moiety (Figure 9). Based on the structure variety of the side chain (R), GLS can be classified into different groups, the most common are aliphatic, *ω*-methylthioalkyl, aromatic, and heterocyclic (indole) GLS (Fahey, Zalcmann, and Talalay 2001).

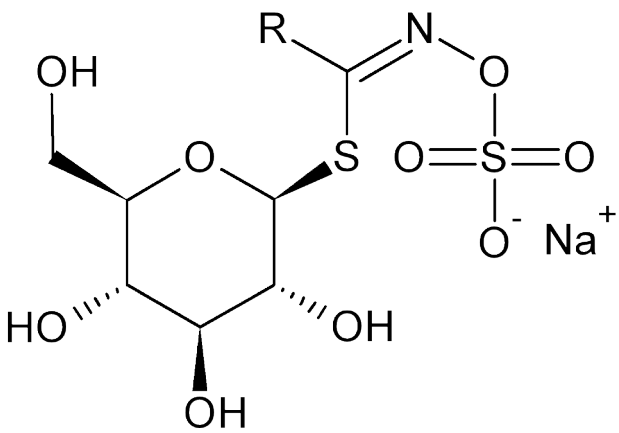



**Figure 9.** General structure of glucosinolates. Adapted from www.wikipedia.org.

According to previous studies, eight different GLS have been found in horseradish (Figure 12b), in which sinigrin (2-propenyl or allyl glucosinolate) and gluconasturtiin (phenethylglucosinolate) were found in higher quantity compared with the others. Sinigrin is the main GLS found in horseradish and accounts for 74% of the total GLS in the plant material (Li and Kushad 2005). The quantity and quality of plant GLS content are significantly affected by the plant's age and environmental factors (e.g. soil fertility, wound, pathogen challenge, etc…) (Björkman et al. 2011).

### 1.2. GLSs hydrolysis reaction

GLSs hydrolysis is triggered when damage is done to the plant. This reaction requires an important enzyme called myrosinase, which is separated under normal conditions to avoid contact with GLS. Once hydrolysis is triggered, myrosinase is released and reacts with GLS, resulting in an unstable aglucone (thiohydroximate *O* sulfonate), which then gives rise to different products depending on reaction conditions and participation of other factors. For example at pH 7, 37–45 °C, and under the effect of myrosinase, the products of GLS hydrolysis reaction are mainly isothiocyanates (ITCs). Nitrile and epithionitrile are final products if the reaction takes place at pH 3 and 6, respectively, in the presence of $Fe^{2+}$ ions and an epithiospecifier protein. The participation of thiocyanate-forming factor in the reaction results in the formation of thiocyanate. ITC, thiocyanate, nitrile, epithionitrile, and oxazolidinethione are possible products of GLS hydrolysis, of which ITC has recently attracted research, as a potential anticarcinogenic agent (Zhang 2010; Li and Kushad 2005).

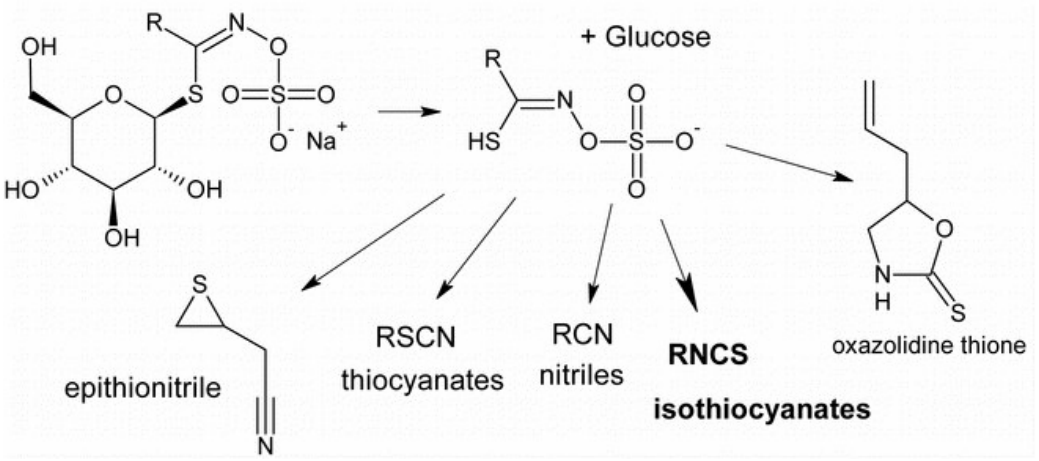



**Figure 10.** Glucosinolates break-down pathways. Figure was made by Marvin JS version 16.9.12.

### 1.3. Isothiocyanates (ITCs)

*Structure and properties* – The most common products yielded from hydrolysis of GLSs are ITCs that share a common structure consisting of –NCS group and side chain –R (figure 11). Because of the –NCS group, specifically the C atom of the group, ITCs possess electrophilic characteristics, i.e. they favor reactions with nucleophilic molecules (e.g. cellular peptides and amino acids), thus leading to possible pharmacological effects. Electrophilicity is influenced by the side chain –R because of the steric hindrance effect on the electrophilic C atom. In addition to electrophilicity, the side chain also influences the lipophilicity of the molecule (Zhang 2004). ITCs are more stable in acidic conditions than in neutral or alkaline conditions due to their reactions with water molecule's OH− ions (Ohta, Takatani, and Kawakishi 1995). Organic solvents (e.g., hexane, acetone, and ethyl acetate) are more favorable for storage than aqueous solutions, in which decomposition of ITCs is temperature

dependent. The decomposition rate is fast at temperature 37°C, decreasing and stopping as the temperature decreases to −5°C. The ability to react readily with ethanol makes ITCs unstable in alcoholic solution. Addition of citric acid, sugar esters, or vegetable oil may stabilize a solution of ITCs (Ina et al. 1981).

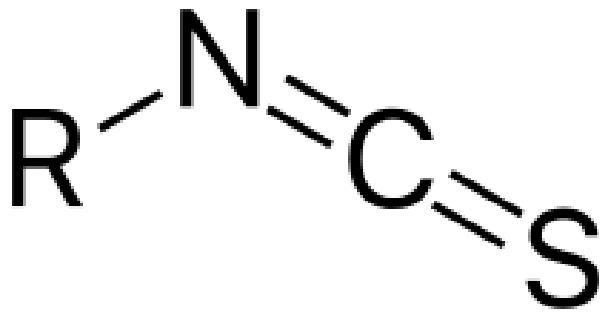
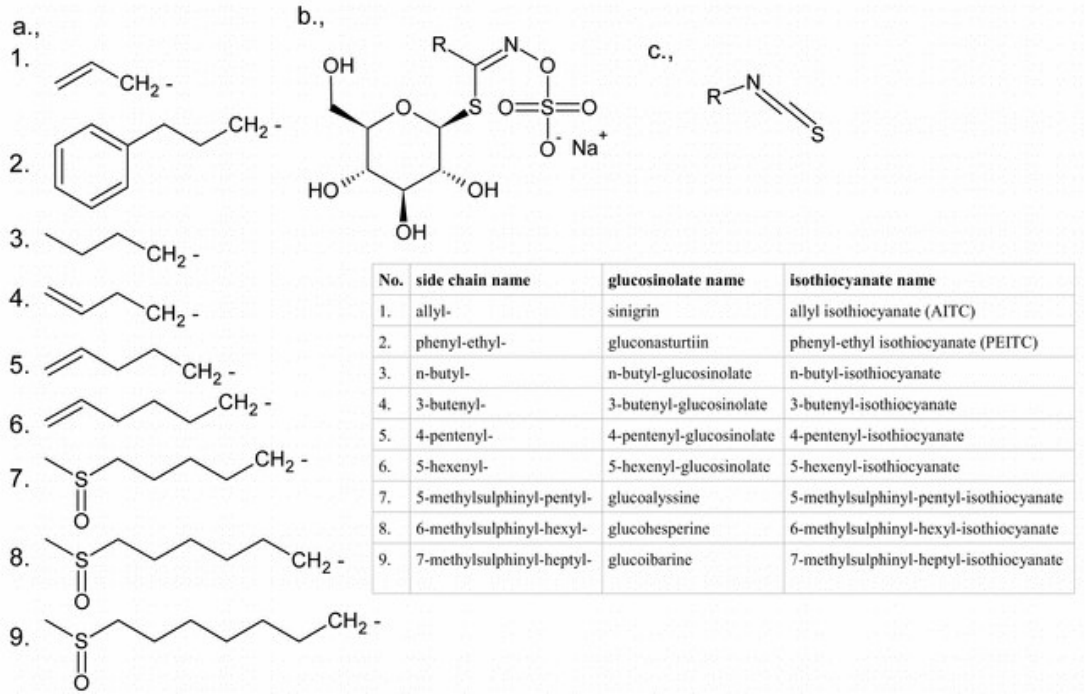



**Figure 11.** General structure of Isothiocyanate. Adapted from www.wikipedia.org.

*Horseradish ITCs* – Allyl ITC (AITC) and 2-phenylethyl ITC (PEITC) are the most common ITC components found in horseradish root. Whereas AITC is also found to be present in both horseradish and wasabi (*Wasabi japonica*), PEITC is only found in horseradish, which may at least partly contribute to the difference in taste between the two species (A. Depree, M. Howard, and P. Savage 1998). AITC constitutes 78% of total horseradish ITCs. AITC is most likely responsible for the pungent, lachrymatory odor and taste of horseradish root. It is the final product from hydrolysis of sinigrin. Also known by the common name "mustard oil," at room temperature AITC appears as a colorless liquid with a boiling point of 150°C and melting point of −80°C (Zhang 2010; E. Y. Yu et al. 2001). PEITC, also referred to as "phenethyl mustard oil", is the colorless or light yellow final product of the hydrolysis of gluconasturtiin.




| No. | side chain name | glucosinolate name | isothiocyanate name |
|---|---|---|---|
| 1. | allyl- | sinigrin | allyl isothiocyanate (AITC) |
| 2. | phenyl-ethyl- | gluconasturtiin | phenyl-ethyl isothiocyanate (PEITC) |
| 3. | n-butyl- | n-butyl-glucosinolate | n-butyl-isothiocyanate |
| 4. | 3-butenyl- | 3-butenyl-glucosinolate | 3-butenyl-isothiocyanate |
| 5. | 4-pentenyl- | 4-pentenyl-glucosinolate | 4-pentenyl-isothiocyanate |
| 6. | 5-hexenyl- | 5-hexenyl-glucosinolate | 5-hexenyl-isothiocyanate |
| 7. | 5-methylsulphinyl-pentyl- | glucoalyssine | 5-methylsulphinyl-pentyl-isothiocyanate |
| 8. | 6-methylsulphinyl-hexyl- | glucohesperine | 6-methylsulphinyl-hexyl-isothiocyanate |
| 9. | 7-methylsulphinyl-heptyl- | glucoibarine | 7-methylsulphinyl-heptyl-isothiocyanate |

**Figure 12.** Main constituents of horseradish root. (a) General structure of glucosinolates (GLS). (b) Side chains of horseradish main glucosinolates and their respective breakdown products, the isothiocyanates. (c) General structure of isothiocyanates (ITCs). Figure was made by Marvin JS version 16.9.12 and Gimp 2.8. Data is adapted from previous study( Zhang 2010).

## 2. GAS CHROMATOGRAPHY AND MASS SPECTROMETRY (GC / MS) STUDY ON HORSERADISH ESSENTIAL OIL

*"Gas chromatography (GC) and mass spectrometry (MS) are highly compatible techniques used for identification and quantitation of volatile and semi-volatile compounds in complex mixture. GC separates volatile / semi-volatile compounds with high resolution, whereas MS reveal detailed structural*

*information of the analytes by fission reaction (fragmentation) and mass to charge (m/z) ratio of resulted ions."* ("GC-MS: Principle, Technique and Its Application in Food Science" 2016)

### 2.1. Sample preparation and method

*Sample preparation* – for studying isothiocyanate profile, 10µl horseradish essential oil from both main and lateral roots, was diluted in 990 µl tert-butyl methyl ether (99% analytical scale, Scharlau). The solution was mixed, centrifuged and transferred to vials ready for GC / MS study. The post-distillation sample mush and water extract were also taken into consideration to determine the efficiency of the distillation method. For these studies, 100mg mush were mixed with 900 µl acetone and ready for direct injection. The water extract were obtained from liquid / liquid extraction 10 times diluted with acetone. The water extract was directly injected.

*Method* – 1µl sample solution was injected in 100:1 split mode to an Agilent 7890A GC 5975C MS instrument, with a HP-5 5% phenyl methyl siloxan HP-5 column (30 m × 320 µm × 0.25 µm column); flow rate was 3 ml/min (helium). Front inlet was heated to 150 °C. Initial oven temperature was 50 °C, held for 3 min; followed by a temperature gradient with rate of 15 °C/min to 200 °C for 2 min, and then 40 °C/min to 320 °C for 5 min (total length of 23 min). Transfer line temperature was set at 320°C, ions were detected in the m/z range 40–500. For quantification of AITC, five-point calibration curves were used with concentration of 0.25, 0.50, 100, 200, 300 mg/ml. OPENChrom 1.1.0 Diels software was used to view spectra. The identification was made based on the characteristics of fragmented ions for each molecule and compared them to the

data from literature (KJZER 1963).

## 2.2. Result and discussions

### *2.2.1. ITC components of horseradish essential oil*

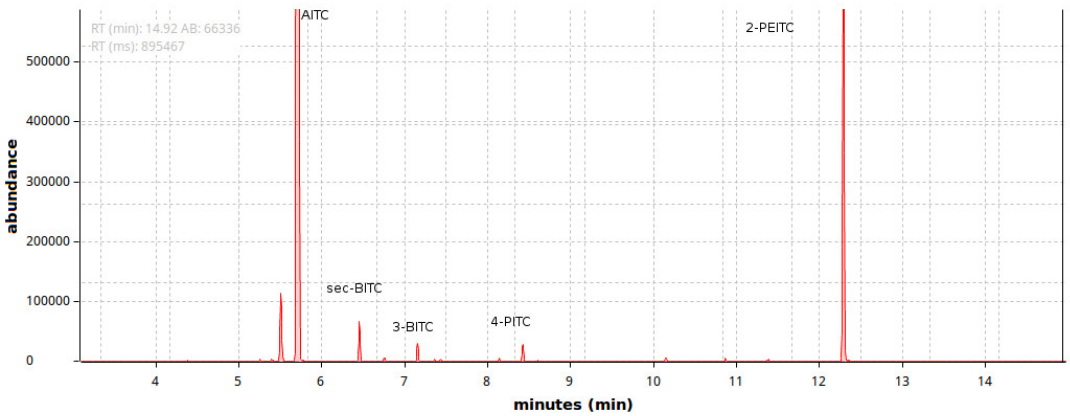



**Figure 13**. Isothiocyanate profile of horseradish essential oil is shown on gas chromatograph viewed by OPENChrom 1.1.0 Diels. Abbreviation – AITC: allyl isothiothiocyanate, sec BITC: sec-butyl isothiocyanate, 3-BITC: 3-butenyl isothiocyanate, 4-PITC: 4-pentenyl isothiocyanate, 2-PEITC: 2-phenethyl isothiocyanate.

Five identified isothiocyanates present in horseradish essential oil are allyl isothiocyanate (AITC), 2-phenethyl isothiocyanate (2-PEITC), sec-butyl isothiocyanate (sec-BITC), 3-butenyl isothiocyanate (3-BITC), and 4-pentenyl isothiocyanate (4-PITC) (figure 13). The characteristics of AITC spectra are at the base peak of m/z = 47 (allyl ion) and 99 ($M^+$ ions). The abundance of allyl ion (m/z = 49) and $M^+$ (m/z = 126) are the signatures of 4-PITC spectrum. Spectra with the presence of $CH_2$=$NCS^+$ ions (m/z = 72) and $M^+$ (m/z = 113)

belongs to 3-BITC. 2-PEITC (M.W = 163), an aromatic ITC, possesses an intensive peak at m/z = 91 due to the present of the stable tropylium ion $[C_7H_7]^+$. Spectra of sec-BITC (M.W. = 115) possess a high peak at m/z = 86 due to the presence of the fragmented ion $CH_3CH{=}NCS^+$ (KJZER 1963). The essential oil extracted (large-scaled extracting) from horseradish from Újléta, Hungary showed major amount of AITC and PEITC, which consolidated other previous studies on ITC profile of horseradish. Together, AITC and PEITC composed of 98% of total ITCs where AITC comprised a higher proportion (85%), similar to collected data from literature. The 5 identified isothiocyanates were also found in previous study on isothiocyanate content of horseradish cultivated in New Zealand (Sultana et al. 2003), in which 8 isothiocyanates were identified. Isobutyl-, 3-methylthiopropyl- and benzyl- isothiocyanates were not found in this study, probably because of their minor amount present in essential oil. However, the amount of allyl isothiocyanate measured in this study was 30% higher than that of the previous study. The AITC content is 2331.57mg/kg fresh horseradish roots compared to 1658.1mg/kg in the other study. In the case of lateral roots, AITC level is only 382.12mg/kg fresh roots. The concentration of AITC was calculated on the calibration curve (Beer's law) according to the sensitivity for AITC.

***Table 1. Characteristic Ion Fragments of ITCs from Horseradish Essential Oil***

| Isothiocyanates (ITC) | Glucosinolate (GLS) | characteristic m/z |
|---|---|---|
| allyl ITC | sinigrin | 99, 41 |
| 2-Phenethyl ITC | gluconasturtiin | 163, 91 |
| Sec-butyl ITC | 1-methyl-butyl-GLS | 86, 115 |
| 3-butenyl ITC | gluconapin | 113, 72, 85 |

| | | |
|---|---|---|
| 4-pentenyl ITC | glucobrassicanapin | 126, 41,72 |

*2.2.2. The efficiency of distillation method*

The efficiency of distillation method is determined by the amount of extracted essential oil, the glucosinolate / isothiocyanate content present in post-distillation sample materials and isothiocyanate content in the watery extract. With the acceptable yield, the distillation is considered as completed based on the results that there was no / little trace of glucosinolate / isothiocyanate in the post-distillation sample mush. I.e. the hydrolysis reaction converting glucosinolate to isothiocyanate is completed (no trace of sinigrin) and the minor amount of AITC (0.003 ± 0.005mg/ml) suggested that the interested component were extracted almost completely from the plant material. The reaction of water with isothiocyanates can influence the isothiocyanate content of extracted horseradish essential oil. The studies on watery extract obtained from the distillation revealed the content of isothiocyanates "trapped" in this medium. It was compared to the isothiocyanate content of extracted oil to evaluate the efficiency of the distillation method. The result showed that very small content of isothiocyanate (approximately 15-20ppm) is present in the watery extract, i.e. very low degree of interaction occurs between the main components and water. These results consolidated that the new distillation method is efficient in extracting essential oil from horseradish roots.

## 3. CAPILLARY ELECTROPHORESIS (CE) STUDY

*"Capillary electrophoresis is the technique for separation of molecules, which employs narrow bore capillaries and high applied voltage. Due to the high*

*voltage application, electroosmotic and electrophoretic flows of buffer solution / ionic species are generated within the capillary, which facilitate the separation of both large and small molecules. Micellar electrokinetic capillary chromatography (MEKC) is excellent CE mode for determination of small molecules and separation of both charged and uncharged molecules. MEKC employs the use of surfactants. When the concentration of surfactants is above critical micelles concentration (CMC), micelles are formed by the aggregation of these amphiphilic molecules, which can organize analytes based on hydrophobic and electrostatic interactions with micelles." (Tagliaro et al. 1998)*

### 3.1. Material preparation and instrument

*The sample preparation* – Fresh vegetables (i.e., Brussels sprouts, horseradish, radish and watercress) used for the study were obtained from local suppliers. Plant material (approximately 15 g) was homogenized in commercial grinder after addition of 15.0 mL 20 mM phosphate buffer (pH 6.50; Reanal, Budapest, Hungary) at 4 °C. The paste-like sample was centrifuged at 20000 rpm for 5 minutes, filtered (0.20 μm pore size), and the supernatant was ready for activity measurements. Dilutions (if necessary) were done with the extracting buffer. The plant extracts were always made fresh, and stored at 4 °C before being added to the enzyme assay. If the myrosinase had to be inactivated, an aliquot of the extracts containing active myrosinase was put in a test tube and the tube was immersed in boiling water for five minutes. This was used for accuracy measurements, as a "plant matrix".

For glucosinolate determinations, the plant material (approx. 10 g) was

immersed in boiling water for 10 minutes (watercress) and 30 minutes (other vegetables) to completely inactivate the myrosinase. 10 mL of MeOH was added to the cooked plant material, followed by thorough homogenization, and centrifugation at 13000 rpm for 3 minutes. The supernatant was evaporated to dryness. Prior to analysis, the dried samples were resuspended in water, centrifuged and subjected to analysis by both CE and LC/MS - after dilution with water, if necessary. These extracts are referred to as "methanolic extracts".

*The instrument* – Method development was carried out on a PrinCE-C 700 capillary electrophoresis instrument. A 60 cm fused silica capillary with 50 μm (i.d.) was used. For myrosinase activity study, effective length was 7.2 cm (short-end injection). For determination of different glucosinolates or allyl isothiocyanate quantification from concentrated real matrices, effective length was 52.8 cm (long-end injection). Capillary preconditioning and post-conditioning were previously described (Gonda et al. 2013), Sample injection was hydrodynamic (100 mbar × 0.25 min.). Sinigrin was quantified at 230, gluconasturtiin at 210, ITC derivates at 275 nm.

The softwares – ChemAxon MarvinSketch was used for drawing chemical structures and reactions, while Calculator Plugins were used for structure property prediction and calculation. Version 6.2.3_b915, 2014 was used. Figures were generated using scripts in R 3.1.1. (R Development Core Team, 2009) using ggplot 0.9.3.1.

## 3.2. Method

### *3.2.1. Optimization of separation*

The basic of the new method is to be able to separate GLSs and ITCs from the plant matrix. Solutions of pure sinigrin (SIN), gluconasturtiin (GNT) and methanol extract of horseradish roots were used. The starting background electrolyte (BGE) solution contained N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS, 20mM), sodium deoxycholate (250 mM), sodium tetraborate (15 mM), pH was 8.50. Influencing parameters (i.e., pH, concentration of electrolytes and surfactants, addition of organic solvents and polarity (short-end injection mode)) were taken into consideration in order to improve the speed of the operation with similar sensitivity and stability.

*3.2.2. Derivatization study*

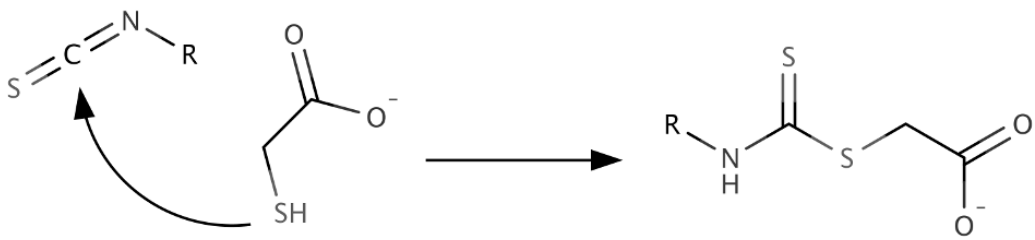



**Figure 14.** Derivatization scheme of isothiocyanates generated in-vial by myrosinase mediated decomposition of glucosinolates. Figure was made by Marvin JS version 16.9.12.

Dithiocarbamate is the product of in-vial derivatization reaction of ITCs with mercaptoacetic acid (MAA) (figure 14). MAA is the suitable derivatizing agent for ITCs because it is inexpensive, miscible with water at any pH, and the product (dithiocarbamate) carries charges at the pH of BGE (pH = 9.0). The

study of optimal concentration of ascorbic acid (enzyme activator) and MAA was designed as following: 350 µg/mL AITC was derivatized in phosphate buffer (10 mM, pH 7.5) with the different concentrations of MAA (1, 5, 10 mM), and ascorbic acid (0, 1, 5, 10 mM). The working pH range of the reaction was investigated based on the quantification (using proposed CE-MEKC method) of the generated derivatized products in different pH – 350 µg/mL AITC was derivatized with MAA (5 mM), ascorbic acid (1 mM) in a pH series of 5.5-9.5 (acetate, phosphate or borate buffers in 10 mM end-concentration). The characterization of derivatization products by LC-MS was run on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS (column: Hypersil Gold 50 mm × 2.1 mm × 1.9µm). ESI ionization parameters were as follows: heater temperature, 300 °C; sheath gas, $N_2$; flow rate, 20 arbitrary units (arb); aux gas flow rate, 8 arb; spray voltage, 4 kV; capillary temperature, 275 °C; capillary voltage, -28.00 V, negative ion mode. Gradient components were A, water with 0.1% (v/v) formic acid; B, MeCN with 0.1% (v/v) formic acid. The time program was 10% B: 0 – 2 min, 10 – 90% B: 2 – 7 min, 90%B: 7-13 min, 90 – 10% B: 13 – 13.1 min, 10% B: 13.1 – 15 min. Flow rate was 300 µL/min. 1 µL of a derivatized sample (acetate buffer, pH 5.0, mercaptoacetic acid (5 mM), ascorbic acid (1 mM)) containing 1 µg/mL of allyl isothiocyanate and phenethyl isothiocyanate was injected.

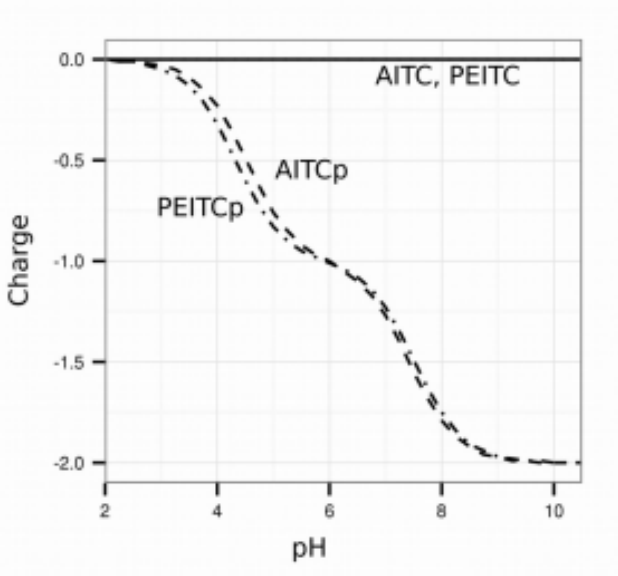



**Figure 15.** Charge of isothiocyanates and their mercaptoacetic acid dithiocarbamate products in the pH range usually used for capillary electrophoresis. Calculations were done by ChemAxon MarvinSketch v6.2.3., using default method parameters. Abbreviations – AITC: allyl isothiocyanate; PEITC: phenethyl isothiocyanate; AITCp allyl isothiocyanate dithiocarbamate product; PEITCp: phenethyl isothiocyanate dithiocarbamate product.

*3.2.3. Validation*

The validation of the new CE method was performed using short-end injection mode. For AITC, the 5-points calibration curve (4.5, 9, 45, 90, 450 µg/ml) was prepared and measured as following: 25 mg/ml AITC stock solution was prepared with MeCN, which then diluted with water to reach the desired concentration. These solutions were mixed with derivatization solution ($NaH_2PO_4$ (100 mM), mercaptoacetic acid (50 mM), ascorbic acid (10 mM), pH 7.50, following 9:1 ratio). In case of sinigrin and gluconasturtiin , 7-point

calibration curve (5, 10, 50, 100, 500, 1000, 5000 µg/ml) was prepared by dilution of these glucohydrolates with water. From the calibration curves, limit of detection (LOD), limit of quantitation (LOQ), coefficient of determination ($R^2$) and regression equations were calculated. Reproducibility studies were designed as following: five injections of isothiocyanate derivatized solution (100 µg/ml), and 100µg/ml glucosinolate solution were introduced and measured per day in 3 days. The relative standard derivation (RSD) between the area under curve (AUC) and retention time was calculated by DAx 8.1. software. For accuracy study, sample of 10 µl inactivated cold buffer horseradish extract/100 µl volume was separately spiked with 1000 µg/ml standards and the recoveries were calculated. The absence of residual glucosinolates in these plant extracts was confirmed by injecting samples without adding glucosinolate standards.

*3.2.4. Quantification of GLSs and AITC from real plant matrix*

Brussels sprouts, horseradish, radish and watercress methanol extract were measured by capillary electrophoresis (long end injection mode) for glucosinolate profile and LC-ESI-MS for sinigrin and gluconasturtiin comparative study. Glucosinolate determination by LC-ESI-MS was done on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS, column: Kinetex XB-$C_{18}$ (100 × 2.10 mm, 2.6 µm, Phenomenex). Five-point calibration curves of sinigrin and gluconasturtiin in water ranging from 0.5 to 40 µg/mL were used as calibration curves. Gradient components were A, water with 0.1% (v/v) formic acid; B, MeCN with 0.1% (v/v) formic acid. The time program was 5% B: 0 – 1 min, 5 – 25% B: 1 – 4 min, 25 – 60% B: 4-5 min, 60 – 5% B: 5 – 6 min, 5% B: 6-8 min. Flow rate was 250µL/min. 1µL of the

diluted methanol extract was injected, typically 5-100-fold dilutions with water were appropriate. The instrument was tuned automatically for sinigrin to obtain the optimal ESI parameters. ESI ionization parameters were as follows: capillary temperature, 275 °C; source heater temperature, 300 °C; sheath gas, $N_2$; sheath gas flow, 30 arbitrary units (arb); aux gas flow, 5 arb; source voltage, 3 kV; capillary voltage, -1.00 V, negative ion mode.

The new CE method is applied on the study of AITC content of food products (i.e., mustard sauce, two types of horseradish sauces and wasabi cream). The samples were composed of 100 mg of food product diluted with 100µl buffered deodorization solution ( $NaH_2PO_4$ (100 mM), ascorbic acid (10 mM), mercaptoacetic acid (50 mM), pH 7.5) and 800 µl water. After mixing and centrifugation, the supernatant was introduced directly to CE (long-end injection mode) for measurement. pH readjustment (to 7.5) is necessary if the products contain significant amount of vinegar.

*3.2.5. Myrosinase activity determination and AITC release study*

The myrosinase activity study was designed as following: The myrosinase-inactivated plant extract obtained by buffer extraction of boiled plant, served as negative controls. The fresh plant extracts by cold buffer were diluted with buffer solution ( 100 mM $NaH_2PO_4$, 10 mM ascorbic acid, pH 6.50) in 9:1 and water. 5 µL GLS stock (10 mg/ml) was added to 190 µL of previous mixture. Addition of the substrate was the reaction start point, negative controls were obtained by using plant extracts that were previously boiled to inactivate the myrosinase. The reaction was run at 25 °C for 5 minutes, and then terminated by heating the test tubes to 100 °C for 5 minutes, followed by the sinigrin

concentration determination by CE after centrifugation at 13000 rpm for 1 min. Substrate concentration decrease was kept below 10%. The plant extracts were also checked for the presence of residual substrates (no sinigrin added). Protein determination from these extracts was done using Bradford's reagent with bovine serum albumine as standard. Under the same conditions, a series of different initial sinigrin concentrations were tested to obtain the $K_m$ constant for myrosinase. Tested initial concentrations were 20, 35, 50, 65, 100, 150, 225, 300µg/ml, the determination was run in three replicates. The added myrosinase containing 50-fold diluted horseradish extract was allowed to decompose sinigrin for 5, 8.75, 12.5, 16.25, 25, 37.5, 56.25, 75 minutes, respectively, keeping decomposed substrate below 10%, allowing the estimation of the initial reaction rate ($v_0$). The $K_m$ value was calculated by fitting the Michaelis Menten equation ($v_0 = v_{max}[S] / K_m+[S]$) to the obtained data. For non-linear curve-fitting, the nls package in R was used (n=3).

The same vegetable extracts were also assayed for myrosinase activity by the widely used pH stat assay (Piekarska et al., 2013). The reaction mixture was the same as that for CE, except that it was not buffered: to 7.66 mL of water 80 µL of ascorbic acid solution (100 mM, pH adjusted to 6.50 with NaOH), 80 µL of plant extract (diluted if necessary) was added. After the pH drift stopped after a few minutes, the reaction was initialized by addition of the substrate (final concentration: 250 µg/mL). Thereafter, freshly prepared 1 µM NaOH was added under slow constant stirring to keep the pH at 6.50. The amount of NaOH consumed by the released $H^+$ during glucosinolate decomposition was registered for 5 minutes. Extracts of the four vegetables (Brussels sprouts, horseradish, radish, watercress) were compared for the sinigrin aglycon – allyl isothiocyanate conversion rate. The reaction mixture was: 10 µL of buffered

derivatization solution (100mM $NaH_2PO_4$, 10 mM ascorbic acid, 50 mM mercaptoacetic acid, pH 7.5), 60 µL bidistilled water, 10 µL enzyme containing extract (not diluted) and 20 µL of sinigrin stock solution (10 mM). The experiment was designed to result in 2 mM allyl isothiocyanate if the conversion ratio is 100%.

### 3.3. Result and discussion

#### *3.3.1.Optimization of separation*

A good resolution for GLSs (sinigrin / gluconasturtiin) was observed in horseradish methanol extract using the starting BGE and long end injection mode. However, the method could be further developed in the aspects of decreasing measuring time and increasing sensitivity. Completely removing or decreasing the concentration of BGE 's components can shorten the measuring time due to the possibility to increase the voltage. Completely removing of borate and decreasing sodium deoxycholate concentration (from 250 to 175 mM) showed no effect on the resolution between GLSs. Further decrease of analysis time is also achieved by increasing pH to 9.0, i.e., giving the ability to increase electroosmotic flow (EOF). At pH 9.0, CHES (*N*-Cyclohexyl-2-aminoethanesulfonic acid) was used as a buffering agent (significant buffering capacity, low UV absorption). Other strategies such as addition of organic solvents, employing different surfactants (also as mixtures) resulted in loss of resolution and/or sensitivity for some analytes of interest.

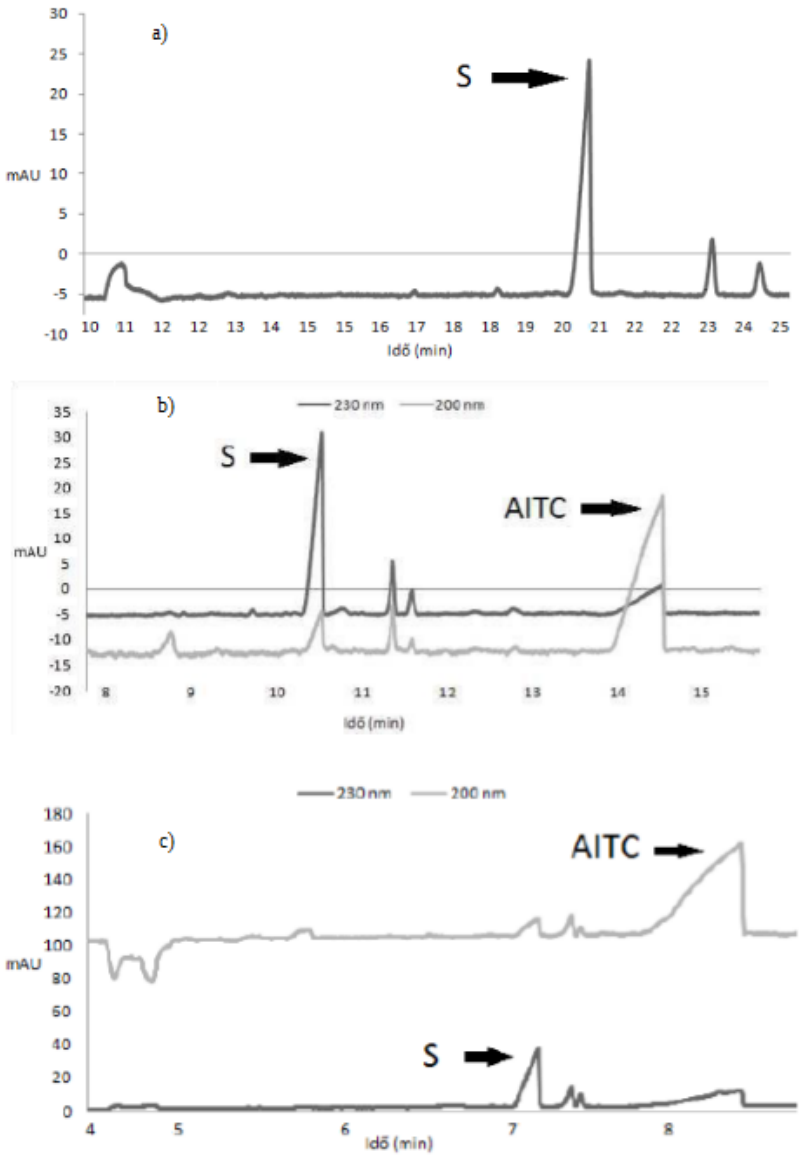



**Figure 16.** Electropherograms of the different methods used during optimization on PrinCE-C 700 capillary electrophoresis. a) Background electrolyte was 20 mM TAPS, 15 mM Borate, 250 mM SDC, pH 8.5. b) Background electrolyte was 20 mM TAPS, 250 mM SDC, pH 8.5. c) Background electrolyte was 20 mM TAPS, 175 mM SDC, pH 8.5.

The most effective BGE contains CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0, applied voltage 20 kV. It showed good resolution for sinigrin / gluconasturtiin, and no major interfering peak in the methanol extract of horseradish. Although AITC was separated from GLSs, because of its low specific absorbance, the limit of detection is so high which will be the problem for further study. Increasing the sensitivity of the method for AITC was the next step for method improvement.

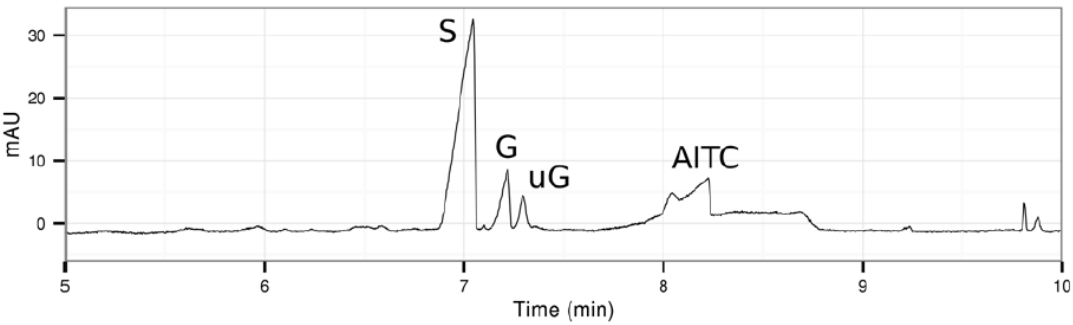



**Figure 17.** Electropherogram of a myrosinase inactivated (cooked) horseradish root extract, spiked with 500 ppm allyl isothiocyanate without derivatization, using the proposed CE-MEKC procedure in long-end injection, with 52 cm effective length. Background electrolyte was 20 mM CHES, 175 mM SDC, at pH 9.0. As detection wavelength, 230 nm was used. Note good resolution between GLSs, and low sensitivity and resolution for the underivatized AITC. Abbreviations: AITC, allyl isothiocyanate; G, gluconasturtiin (phenethyl glucosinolate); S, sinigrin; uG, unidentified glucosinolate.

*3.3.2. Derivatization study*

The MAA concentration was found to influence the amount of the product but not significant: using two-fold concentrations of MAA (10 mM) resulted in 19.7% increase of the average peak area of the product, compared to the proposed 5 mM, thus, this phenomenon did not result in inaccuracy. Similarly, ascorbic acid concentration can influence the reaction but not in significant degree in the tested range (0-1 mM, $p > 0.05$) (Hanschen et al. 2014; Kleinwächter and Selmar 2004). In the study on the influence of pH on derivatization, the reaction gives higher sensitivity between pH 6.5-9.5, and significantly less, but still detectable amounts of products at pH 4.5-5.5. The optimal derivatization solution contains MAA (5 mM), ascorbic acid (1 mM) at pH 7.5, which is suitable for both the activity of myrosinase enzyme and derivatization reaction (Li and Kushad 2005). LC-ESI-MS/MS study in negative ion mode consolidated the presence of the dithiocarbamate product without side products. The most abundant peaks are at m/z of 190 and 254, which corresponded to $[M-H]^-$ ions of dithiocarbamates of AITC and PEITC, respectively. These products are polar at $pH \geq 7$, i.e., they are water soluble and resulted in lower retention time in CE-MECK.

### *3.3.3. Validation*

The characteristics of new developed method are calculated and showed in the tables below. The RSDs of the determination were acceptable, the method showed sufficient stability during the validation. The presence or absence of mercaptoacetic acid in the solution did not influence the characteristics of the glucosinolates, their detection was sensitive and reproducible. Gluconasturtiin behaved very similar to sinigrin during the analysis, it can be reproducibly analyzed with the proposed method.

***Table 2. Validation of New Developed Method for Allyl Isothiocyanate***

| | Sinigrin | Allyl isothiocyanate [a] |
|---|---|---|
| Detection wavelength (nm) | 230 | 275 |
| Linear Regression Equation (ppm) | f(x) = 0.0085 x + 0.0263 | f(x) = 0.0260 x - 0.9750 |
| Linear Regression $R^2$ | 0.9999 | 0.9998 |
| Upper limit of quantification (ppm/mM) | 5000 / 12.58 | 350 / 3.53 |
| LOD (ppm/mM) | 11.93 / 0.030 | 13.50 / 0.136 |
| Retention time RSD% (interday) | 3.07 % | 2.01 % |
| Retention time RSD% (intraday) | 2.02 % | 1.36 % |
| Area under curve RSD% (interday) | 5.51 % | 4.45 % |
| Area under curve RSD% (intraday) | 4.03 % | 4.64 % |

*a : Values Allyl isothiocyanate Data are given as AITC equivalent, but the compound is actually quantified after derivatization with mercaptoacetic acid in-vial, in phosphate buffer (10mM, pH 7.5).*

By using derivatization reaction, the sensitivity for allyl isothiocyanate is increased by about an order of magnitude, making it possible to determine the minute amount of on-line generated ally isothiocyanate for further study. In the case of PEITC, the calibration curves showed low reproducibility, possibly

because of the poor water solubility of the derivatized product. Addition of organic solvent (e.g., 20% MECN) could solve the problem but this is not compatible with enzymatic studies. However, the useful linear range, sensitivity, and intra-day RSD for the gluconasturtiin / PEITC pair was comparable to that of sinigrin, and allyl isothiocyanate, respectively.

***Table 3. Validation of New Developed Method for Phenethyl Isothiocyanate***

| | Phenethyl isothiocyanate [a] |
|---|---|
| Detection wavelength (nm) | 275 |
| Linear Regression Equation (ppm) | f(x) = 0.0093 x + 0.0018 |
| Linear Regression $R^2$ | 1.0000 |
| Upper limit of quantification (ppm/mM)[b] | 450 / 2.78 |
| LOD (ppm/mM) | 13.75 / 0.084 |
| Retention time RSD% (interday) | 0.71 % |
| Retention time RSD% (intraday) | 1.64 % |
| Area under curve RSD% (interday) | 14.9 % |
| Area under curve RSD% (intraday) | 1.87 % |

*a : Values Phenethyl isothiocyanate data are given as PEITC equivalent, but the compound is actually quantified after derivatization with mercaptoacetic acid in-vial, in phosphate buffer (10mM, pH 7.5). b: Values are given for injection with 100 mbar x 0.25 minutes.*

For both pairs of GLS-ITC, the resolution was excellent between the substrate, the product, ascorbic acid and the excess derivatization reagent even in short-end injection mode (figure 18). If desired, an enzyme containing sample subjected to study could be injected about every 4 minutes (figure 19, 20).

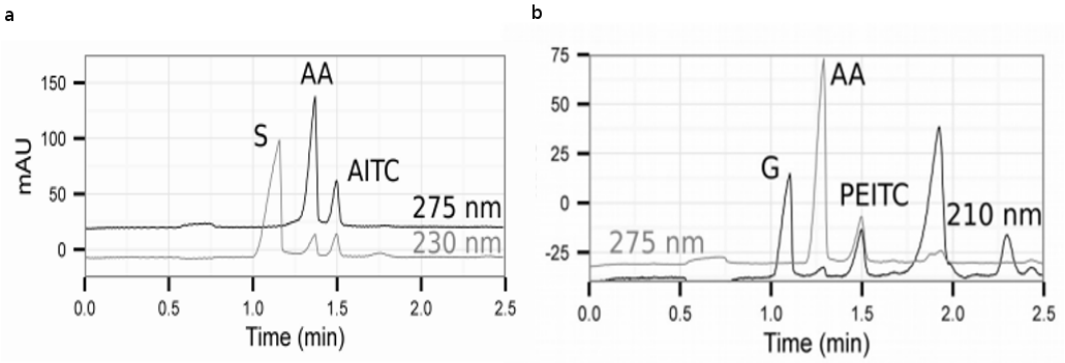



**Figure 18.** Electropherograms obtained using the proposed capillary electrophoresis – micellar electrokinetic chromatography (CE-MEKC) method showing possible applications. Background electrolyte: CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0. a: Isothiocyanates are present as dithiocarbamates during separation. Sample matrices: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), mercaptoacetic acid (5 mM).Standard mixture (sinigrin, allyl isothiocyanate) in derivatization buffer, analyzed in short-end injection mode. b: Close-up of a gluconasturtiin to phenylethyl isothiocyanate decomposition study in short-end injection mode. Abbreviations: AA, ascorbic acid; G,substrate (gluconasturtiin); PEITC, phenethyl isothiocyanate derivatized to dithiocarbamate.

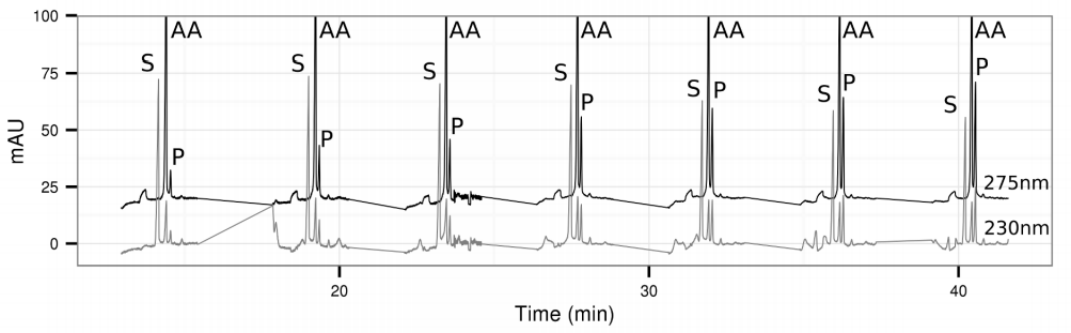


**Figure 19.** Electropherogram series from seven sequential injections of an active myrosinase containing mixture (10-fold diluted radish extract) using the proposed CE-MEKC method. Conditions: short end injection, -20.0kV, BGE: CHES (20 mM), SDC (175 mM), pH 9.5; sample matrix: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), MAA (5 mM). Note decomposition of sinigrin (substrate), increase in the product isothiocyanate derivate concentration, and lack of interfering peaks from the unpurified plant extract. Abbreviations: AA, ascorbic acid; S, substrate (sinigrin); P, product (allyl isothiocyanate derivatized to dithiocarbamate). Excess derivatization reagent (MAA) is detectable, but is not shown, as it passes the detector at the time of the injection of the next sample.

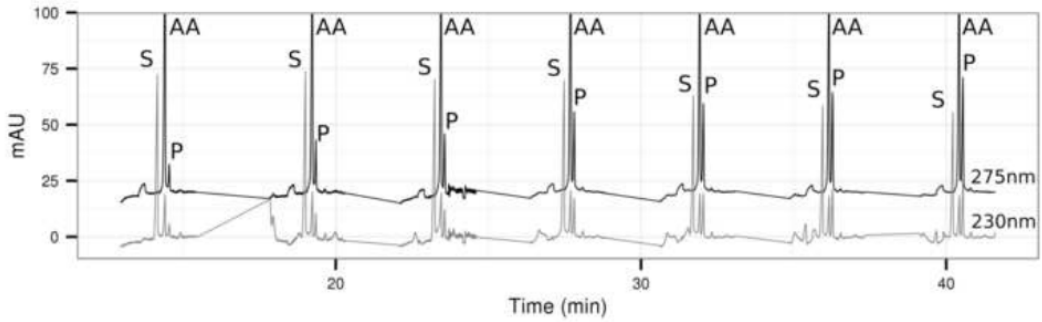



**Figure 20.** Electropherogram series from seven sequential injections of an active myrosinase containing mixture (250-fold diluted horseradish extract) using the proposed CE-MEKC method (Conditions: short end injection, -20.0kV, BGE: 20 CHES, 175 SDC, pH 9.0; sample matrix: 10 mM phosphate, pH 7.0, 1 mM ascorbic acid, 5 mM MAA). Note decomposition of gluconasturtiin (G, substrate), increase in the product isothiocyanate derivate (P) concentration, and lack of interfering peaks from the unpurified plant extract. Abbreviations: AA, ascorbic acid; G, substrate (gluconasturtiin); P,

product (phenethyl isothiocyanate derivatized to dithiocarbamate).

*3.3.4. Determination of glucosinolates and isothiocyanates from plant and food matrices*

The developed method was exploited to quantify sinigrin and gluconasturtiin content of different plant matrices (figure 21). The sinigrin and gluconasturtiin content of four vegetables is summarized in table 4. The values are comparable to that of LC-ESI-MS, the difference between the two methods ranged from 82.3-108.6 %, with an average of 99.6%.

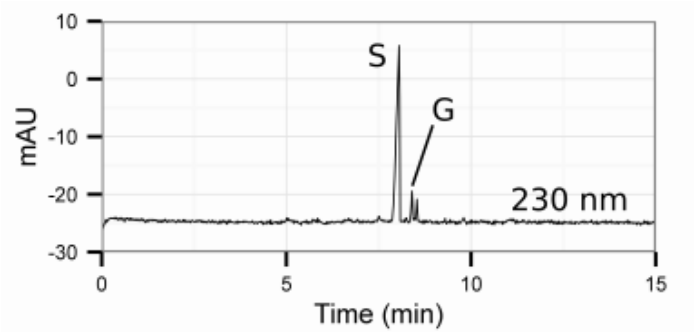



**Figure 21.** Methanolic extract of horseradish root redissolved in water, measured in long-end injection mode. Electropherograms obtained using the proposed capillary electrophoresis – micellar electrokinetic chromatography (CE-MEKC) method showing possible applications. Background electrolyte: CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0. Isothiocyanates are present as dithiocarbamates during separation. Sample matrices: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), mercaptoacetic acid (5 mM).

***Table 4. Glucosinolate Content of Four Tested Vegetables, as Measured by the Proposed CE Method, or by LC-ESI-MS.***

| Vegetable | CE | LC-ESI-MS | CE | LC-ESI-MS |
|---|---|---|---|---|
| | Sinigrin (μg/g (FW)) | | Gluconasturtiin (μg g-1 (FW)) | |
| Radish | n.d | n.d | n.d | n.d |
| Brussels sprout | 161 | 148.2 | n.d | n.d |
| Watercress | n.d | n.d | 162.9 | 153.7 |
| Horseradish | 2291.8 | 2784.4 | 248.5 | 244.3 |

*Abbreviations: CE, capillary electrophoresis; FW, fresh weight.*

The application of proposed method showed the tested commercial condiments contain 369 – 418 μg/g AITC. The method provided a good resolution with simple sample preparation procedure, as plotted in figure 22. The presented long end injection method separates the analytes of interest within 15 minutes from real matrices. This falls in the range of the fastest HPLC methods available for determination of sinigrin and allyl isothiocyanate (Tsao et al. 2002). Typical methods last 20-25 minutes or more, consume 1mL/min solvent and require the removal of protein and fat before analysis (Budnowski et al. 2013; Herzallah and Holley 2012). Direct, simultaneous quantifications from complex matrices can be even longer (Song et al. 2005). Isothiocyanate determination – even when derivatized – can also be time demanding. The presented method is similar in speed to the fastest available CE methods for glucosinolates, an analysis time of 15-25 minutes is typical (Karcher and El Rassi 1999). However, the separation of isothiocyanate adducts usually requires more time (Bjergegaard et al. 1999). With the new method, separation of allyl isothiocyanate dithiocarbamate was also done within 15 minutes. The CE

screening is also faster as compared to GC-MS methods. The usual time of a GC-MS for different isothiocyanates measurement is 30-35 minute (Zhao, Tang, and Ding 2007).

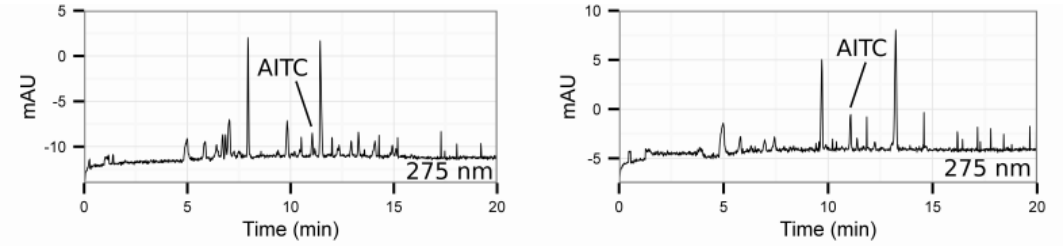



**Figure 22.** Detection of allyl isothiocyanate from food products in long-end injection mode. a., mustard (condiment); b., horseradish sauce with wasabi. Electropherograms obtained using the proposed capillary electrophoresis – micellar electrokinetic chromatography (CE-MEKC) method showing possible applications. Background electrolyte: CHES (20 mM), sodium deoxycholate (175 mM), pH 9.0. Isothiocyanates are present as dithiocarbamates during separation. Sample matrices: phosphate (10 mM, pH 7.5), ascorbic acid (1 mM), mercaptoacetic acid (5 mM).

*3.3.5. Study of myrosinase activity and allyl isothiocyanate release of vegetable extracts*

The average $K_m$ values obtained by non-linear regression analysis of reaction velocity versus sinigrin concentration (at pH 6.50, 25 °C, 0.1 mM ascorbic acid) fell in the range of 0.129 ± 0.025μM. This is the same order of magnitude found for sinigrin in several previous studies for different myrosinases, thus it can be stated that the presented activity is truly that of myrosinase (Li and Kushad

2005; Nehmé et al. 2014).

The activities (expressed in μmol sinigrin decomposed per minute (U)) of tested vegetables are shown in the table below. Activity of sinigrin decomposition ranged from 4.42 U/g fresh weight (watercress) to 208.26 U/g fresh weight (horseradish) in 10 mM phosphate, 1 mM ascorbic acid, pH 6.50, 25 °C, initial substrate concentration: 250μg/ml. The method was shown to be suitable to measure myrosinase activity from low activity mixtures without major interferences. The obtained myrosinase activities with sinigrin as the substrate were compared to those found in the widely used pH-stat assay. Under the same conditions (1 mM ascorbic acid, pH 6.50, at 25 °C, initial substrate concentration: 250 μg/mL), the myrosinase containing extracts of the vegetables had very similar activity (93.7% – 116.9%, average: 107.1%, table 5).

***Table 5. Myrosinase Activities of Four Tested Vegetables Using Sinigrin or Gluconasturtiin as Substrate, Measured By the Proposed CE Method and pH Stat Assay***

| Vegetable | Sinigrin, CE (U/g FW) | Sinigrin, CE (U/mg protein) | Sinigrin, pH stat (U/g FW) | Sinigrin, pH stat (U/mg protein) |
|---|---|---|---|---|
| Radish | 10.31±1.31 | 3.41±0.43 | 11.00±0.94 | 3.64±0.31 |
| Brussels sprout | 7.72±0.6 | 0.96±0.07 | 6.83±0.24 | 0.85±0.03 |

| | | | | |
|---|---|---|---|---|
| Watercress | 4.42±0.5 | 0.76±0.09 | 4.22±0.94 | 0.73±0.16 |
| Horseradish | 208.26±42.94 | 27.69±5.71 | 178.13±4.42 | 23.69±0.59 |

| Vegetable | Gluconasturtiin, CE (U/g FW) | gluconasturtiin, CE (U/mg protein) | S/G AR* | ITC release (%) |
|---|---|---|---|---|
| Radish | 5.35±1.02 | 1.77±0.34 | 1.93 | 92±4.39% |
| Brussels sprout | 3.71±0.09 | 0.46±0.01 | 2.08 | 73.13±0.27% |
| Watercress | 6.01±0.12 | 1.04±0.02 | 0.73 | 102.13±0.94% |
| Horseradish | 197.94±33.98 | 26.32±4.52 | 1.05 | 98.25±3.02% |

*One unit (U) of activity is defined as 1µmol per minute. Abbreviations: CE, capillary electrophoresis; FW, fresh weight; S/G AR: Ratio of myrosinase activity with sinigrin as the substrate / gluconasturtiin as the substrate. The presented values are mean ± SD of three measurements.*

Comparing the activity ratio of the same extracts with sinigrin and gluconasturtiin as the substrate, some level of specificity can be found: Brussels sprouts that contains sinigrin but no gluconasturtiin (Table 5) had 2.08-fold activity against sinigrin as compared to gluconasturtiin. Watercress containing gluconasturtiin but no sinigrin (Table 5) also showed some specificity towards its own glucosinolate. Horseradish, which contains both glucosinolates (Table 5), decomposed both with similar efficacy. Though the measured specificity is not that striking as it was previous described for *Crambe abyssinica*, it suggests that using sinigrin as the sole substrate for myrosinase activity determination may sometimes result in serious under- or overestimation of the biologically

relevant myrosinase activity of plant extracts. Therefore, if possible, methods capable of using substrates other than sinigrin should be integrated into myrosinase activity tests. The phenomenon clearly requires more in-depth study, for which the current method can be used.

### *3.3.6. Discussion*

There are popular methods for myrosinase measurement, most of them are based on spectrophotometry instead of chromatographic separation. These include the indirect measurement of the glucose released (Wilkinson, Rhodes, and Fenwick 1984) or measuring the breakdown kinetics of the substrate (decrease of absorbance maximum). It is also possible to use the released $H^+$ for quantification in a pH-stat assay (Piekarska et al. 2013). As glucosinolate absorbance maxima are usually around 210-230 nm, many compounds can interfere with quantification in UV-Vis, especially when using more concentrated raw extracts. Higher specificity can only be achieved by subjecting the reaction mixture to chromatographic separation. For this purpose, the CE methods can be used. They operate with a minimal amount of sample and are able to study decomposition of different glucosinolates, but neither can give information on the ITC release rate that is of primal biological significance. HPLC methods have excellent reproducibility and sensitivity, but are frequently time consuming and require much more reagents than CE measurements. The reaction mixtures used in this study are simple enough to use short-end injection, which results in less, but sufficient resolution, and less analysis time. In this case, the presented method is capable of separating a glucosinolate – isothiocyanate pair from the reagents in 2.5 minutes. With a capillary reconditioning applied after every sixth injection, a number of about

twelve injections per hour can be reached. This is comparable to the widely used assays' time demand, yet, chromatographic separation takes place, which gives the least interferences from for example ascorbic acid.

For many vegetables, conversion of sinigrin to allyl isothiocyanate was not found to be 100% in the literature. The isothiocyanate yield from the glucosinolate aglycon can range from a few percent to near 100% (Piekarska et al. 2013). This is usually attributed to the presence of specifier proteins that cause the glucosinolate aglycon to rearrange into different volatile products such as nitriles, epithionitriles, thiocyanates among others. In the current study, four vegetable extracts (Brussels sprouts, horseradish, radish, watercress) were successfully compared for ITC conversion rate, with minimal amount of reagents. The procedure also does not require laborious sample preparation (e.g. liquid-liquid extraction) needed to study the ITC content by GC-MS as in previous studies. The amount of allyl isothiocyanate generated from the same amount of added sinigrin (1mM) significantly differed among the vegetable extracts ($p<0.05$, n=3, ANOVA). Conversion rate was found to range between 73.13±0.27% and 102.13±0.94%. The HPLC method successfully separates allyl isothiocyanate and sinigrin from model matrices with active myrosinase within 6 minutes, and was successfully used to measure ITC release (Vastenhout et al. 2014). However, it was not tested for the ability to measure myrosinase activity or ITC release from real matrices, and operates with a 1 mL/min solvent flow. Testing of real matrices would also require the removal of protein and fat as sample preparation steps. The advantages of the proposed CE method include saving time and solvents as compared to many HPLC methods. Hence, it is suitable as a screening method for glucosinolates and allyl isothiocyanate. The method was used as a higher specificity myrosinase assay

that also allows quantification of on-line generated isothiocyanates, the main bioactive products. Only 25-50 μg glucosinolate per sample is sufficient for a myrosinase study, which is especially important in the case of glucosinolates other than sinigrin. Analysis of factors affecting the glucosinolate – isothiocyanate conversion rate is also possible.

# Chapter IV

## Comparative Analysis Of *Armoracia rusticana* And *Armoracia macrocarpa*

## 1. BACKGROUND INFORMATION

### 1.1. Myrosinase

*Myrosinase' s structure and properties* – As mentioned earlier, myrosinase plays a crucial role in the hydrolysis reaction of GLS. Horseradish myrosinase (β-thioglucoside glucohydrolase) is an S-glucosidase enzyme whose (β/α) 8-barrel structure consists of two similar subunits with a molecular weight of 65 kDa linked by a zinc atom (Zhang 2010). To make contact with GLS, horseradish myrosinase requires a hydroxyl group on C2 of the glucose moiety of GLS and a nucleophilic glutamate for catalytic activity (Fahey, Zalcmann, and Talalay 2001).

In *Brassicaceae*, myrosinases are generally classified based on the place where they can be found in the plant. There are three subgroups in this enzyme family, myrosinase A, myrosinase B, and myrosinase C (MA, MB, and MC, respectively). MA and MC can be found only in the seed tissue, whereas MB can be found in most tissues of the plant. Considering this point, horseradish myrosinase should belong in the MB subgroup but classification gets more complicated because horseradish myrosinase possesses a water-soluble property, whereas MB myrosinase does not. This leads to the hypothesis that horseradish myrosinase may belong to an unidentified subgroup of the myrosinase family. Myrosinase is not substrate specific. Horseradish myrosinase not only catalyzes the hydrolysis of GLSs found in horseradish such

as sinigrin, but can also catalyze hydrolysis of GLSs found in different other species (e.g. in broccoli), with rates depending on the substrate (Li and Kushad 2005).

*Myrosinase' s activities* – The optimum conditions for activity of myrosinase are when the temperature of the reaction falls in the range of 37–45°C and pH in the range of 5–8. Horseradish myrosinase activity was observed to increase at 23°C, remaining high (i.e. >80% of maximum) from 37°C, reaching its maximum at 45°C, decreasing at temperatures over 50°C, and the enzyme becomes inactive at temperatures above 70°C. Within the pH range, increasing myrosinase activity was noticed from a pH of 3–4, reaching maximum at pH 5.7 and the activity remains high (i.e. >80% of maximum) at pH range 5–8. Addition of 0.5mM ascorbic acid can significantly stimulate horseradish myrosinase, possibly due to a conformation change in the enzyme leading to increased substrate affinity of horseradish myrosinase and reaction velocity (Bones and Rossiter 2006). Loss in endogenous ascorbic acid was also suggested to cause the decrease of myrosinase activity (A. Depree, M. Howard, and P. Savage 1998). In another study on myrosinase isolated from horseradish grown in China, the best condition for myrosinase activity is at temperature of 65°C and pH 4.0, with ascorbic acid (2 mg/g powdered root) added and an incubation period of 120 min (Zi-Tao Jiang 2006). The inconsistency on the details required for optimal activity between these two studies may be related to differences in geographic regions where the horseradishes were grown. However, it can be concluded that temperature, pH, and ascorbic acid content are the main factors affecting the activity of the enzyme.

**1.2. Peroxidase**

*Structure and properties* – Apart from myrosinase, horseradish contains another enzyme that has gained interest: horseradish peroxidase (HP) – a heme-containing enzyme that utilities hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds, due to its larges-scaled commercial uses, for example as a reagent for organic synthesis and bio-transformation, as in coupled enzyme assays , chemiluminescent assays, immunoassay and the treatment of waste water.

Fifteen HP isoenzymes have been identified from horseradish root. Based on their isoelectric point values, these HPs are referred to by codes as A1-3 (acidic), B1-3 and C1-C2 (neutral basic) and E1-E6 (basic). Among those, the C isoenzyme is the most abundant. Horseradish peroxidase isoenzyme C (HRPC) comprises a single polypeptide of 308 amino acid residues. The structure of the enzyme is largely α-helical and small region of β-sheet. HRPC contain the heme group (iron (III) protoporphyrin IX), located between the distal and proximal calcium binding domains. These metal centers are crucial for the structural and functional integrity of the enzyme. Although so little is known about the function of HP in plant, it is believed to be involved in the conversion of hydrogen peroxide to water and used by plants to regulate level of intracellular hydrogen peroxide. The radical products from HRP-calalysed reactions possibly involve cross-linking reactions (e.g. the formation of diferulate linkages from polymer-attached ferulate groups of polysaccharides or pectins, the formation of dityrosine linkages, etc.), which may be expressed in response to external factors such as the sounding of plant tissue. Water loss and invasion by pathogens can therefore be limited by the formation of a protective polymeric barrier such as suberin (Veitch 2004).

*Horseradish peroxidase's activities* – Differences in activity of HP are observed in different plant tissues and depend on the presence of other substances. HP enzyme activity is observed in an amount of about 49% of total activity in the tap-root, 37% in the lateral root, and 14% in the leaf petiole, with no activity in the leaf blade (Bruno and Njar 2007). Increases in enzyme activity were observed with the presence of other substances such as kanamycin (Tang and Zhang 2005).

## 2. LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC-MS) STUDY ON GLUCOSINOLATES

The aim was to identify glucosinolate composition in plant extracts from both horseradish and its relative *A. macrocarpa* by exploiting the LC-MS method described in previous study (Cataldi et al. 2010). To provide information of GLSs' structures, precursor ion isolation within the linear ion trapping cell and subsequent fragmentation were induced by infrared multiphoton dissociation (IRMPD) as previous studies showed that GLSs form characteristic fragments during $MS^2$ analysis by ion trap and mass spectrometers (Agneta et al. 2012).

### 2.1. Materials and method

*Plant material and extract preparation* – the plant material (approximately 10 g) was immersed in boiling water for 30 minutes to completely inactivate the myrosinase. 10 mL of MeOH was added to the cooked plant material, followed by thorough homogenization, and centrifugation at 13000 rpm for 3 minutes. The supernatant was evaporated to dryness. Prior to analysis, the dried samples were resuspended in water, centrifuged and subjected to analysis by LC/MS -

after dilution with water, if necessary.

*Instrumental* – LC-ESI-MS was done on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS, column: Kinetex XB-$C_{18}$ (100 × 2.10 mm, 2.6µm, Phenomenex). ESI ionization parameters were as follows: capillary temperature, 275°C; source heater temperature, 300 °C; sheath gas, $N_2$; sheath gas flow, 30 arbitrary units (arb); aux gas flow, 5 arb; source voltage, 3 kV; capillary voltage, -1.00 V, negative ion mode.

The spectra were compared to the literature to identify or confirm the presence of certain GLSs.Glucosinolates were identified based on their precursor ions $[M - H]^-$ and characteristic fragmented ions, i.e., exact m/z 96.96010, 195.03327, 259.01292, 274.99008, and 241.00236, which correspond to the fragment ions from the glycone side chain, namely, $HSO_4^-$, $C_6H11O_5S^-$, $C6H_{11}O_9S^-$, $C_6H_{11}O_8S_2^-$, $C_6H_9O_8S^-$, respectively (Agneta et al. 2012). In the case of *A. macrocarpa*, in addition to the use of the precursor ions (for those present in horseradish), a second method was necessary to identify glucosinolate profile due to the lacking of information on glucosinolate composition of this species. The typical fragmented ions (with exact m/z of 259.013, $C6H_{11}O_9S^-$) were used to traced back to their precursor ions. The satisfying spectrum must consist of those fragmented ions which are characteristic for glucosinolate.

**2.2. Results and discussion**

$[M-H]^+$ parent ions of 16 glucosinolates identified in previous study were observed. However, only 6 glucosinolates, which have matched fragments' spectra, were found in this study (table 6). These glucosinolates include

sinigrin, gluconasturtiin, glucobrassicin, glucoiberin, glucocochlearin, and glucoconringianin. Compared to isothiocyanate profile of horseradish essential oil, allyl isothiocyanate and 2-phenethyl isothiocyanate are the products of sinigrin and gluconasturtiin, respectively. The traces of parent glucosinolates of other isothiocyanates present in horseradish oil were found without matched spectra (glucobrassicanapin, gluconapin and 1-methyl butyl glucosinolate). Isothiocyanate products of identified glucosinolates – glucobrassicin, glucoiberin, glucocochlearin, and glucoconringianin were not found in the horseradish oil. This can be explained by the minor amount of these glucosinolates present in horseradish.

***Table 6. Glucosinolates Identified in Horseradish Roots by LC-MS***

| Compound | Precursor ion | Main MS/MS product ions |
|---|---|---|
| Glucoiberin | 422.02549 | 358.02771; 274.9930; 259.01318; 241.00256; 195.9751 |
| Sinigrin | 358.02720 | 274.99030; 259.01327; 241.00223; 195.03329; 179.99740; 161.98670; 116.01759 |
| Glucocochlearin | 374.05850 | 294.10189; 274.99009; 259.01321; 241.00232 |
| Glucoconringianin | 374.05850 | 294.10189; 274.99009; 259.01321; 241.00232 |
| Glucobrassicin | 447.05375 | 274.99035; 269.02430; 259.01349; 241.00280; 205.04450 |
| Gluconasturtiin | 422.05850 | 342.10164; 274.99031; 259.01316; 244.02839; 241.00356; 195.03394 |

In *A. macrocarpa*, 17 tentatively identified GLSs are glucoalyssin,

glucoberteroin, glucolesquerelin, gluconasturtiin, 4-methoxyglucobrassicin (or neoglucobrassicin), glucobrassicin, 1/2/3-methylbutyl GLSs (or n-pentenyl GLS), glucoerucin, 4/5-hydroxyglucobrassicin, 5-hexenyl GLS, glucocochlearin, glucoconringianin, glucosativin, glucoibarin, glucocapparilinearisin (or glucobrassicanapin), glucoarabishirsutain. Among these, 10 glucosinolates with matched spectra in *A. macrocarpa* were also found in *A. rusticana* compared to the literature. These glucosinolates are glucocochlearin, glucoconringianin, glucosativin, glucoibarin, 4-hydroxyglucobrassicin, 5-hydroxyglucobrassicin, glucobrassicin, gluconasturtiin, 4-methoxyglucobrassicin, and glucoarabishirsutain. The other tentatively 7 glucosinolates found only in *A. macrocarpa* are glucoberbertoin, glucolesquerellin, glucoerucin, 1-methyl butyl glucosinolate, n-pentyl glucosinolate, 5-hexyl glucosinolate and glucoalyssin.

Results of this study showed 5 glucosinolates present in both *A. rusticana* and *A. macrocarpa* with matched spectra for each glucosinolate (gluconasturtiin, glucobrassicin, glucocochlearin, glucoconringianin and glucoibarin). Gluconasturtiin is one of those main glucosinolate components in *A. rusticana*. However, the other main glucosinolate – sinigrin is absent in *A. macrocarpa* (table 7). The absence of sinigrin could be contributed to the difference of smell and taste of these two species. The difference of smell and taste could also be due to the amount of GLSs, which required further quantification study for the content of these molecules.

***Table 7. Comparative Table on Glucosinolate Profile of A. rusticana, A. macrocarpa and Data from Literature***

| Glucosinolate | *A. rusticana* | *A.* |
|---|---|---|

| | | | | *macrocarpa* |
|---|---|---|---|---|
| 1 | Glucoiberin | | | |
| 2 | Sinigrin | +/+ | | |
| 3 | 2-methylsulfonyl-oxo-exyl-GLS | | | |
| 4 | Gluconapin | | | |
| 5 | Glucococchlearin | +/+ | | +/+ |
| 6 | Glucoconringianin | +/+ | | +/+ |
| 7 | Glucosativin | | | +/+ |
| 8 | Glucoibarin | +/+ | | +/+ |
| 9 | 4-hydroxyglucobrassicin | | | +/+ |
| 10 | 5-hydroxyglucobrassicin | | | +/+ |
| 11 | Glucobrassicanapin | | | |
| 12 | Glucotropaeoin | | | |
| 13 | Glucobrassicin | +/+ | | +/+ |
| 14 | Gluconasturtiin | +/+ | | +/+ |
| 15 | 4-methoxyglucobrassicin | | | +/+ |
| 16 | Glucoarabishirsutain | | | +/+ |
| 17 | Glucorberteroin | | | +/- |
| 18 | Glucolesquerellin | | | +/- |
| 19 | Glucoerucin | | | +/- |
| 20 | 1-methyl butyl GLS | | | +/- |
| 21 | n-pentyl | | | +/- |
| 22 | 5-hexyl GLS | | | +/- |
| 23 | Glucoalyssin | | | +/- |

*Abbreviation – +/+: identified GLS with matched fragments' spectra; +/-: tentatively identified GLS; GLS: glucosinolate.*

## 3. ANATOMICAL STRUCTURE STUDIES

*"Most biological tissues have very little contrast, and cellular details are hard to discern with the ordinary light microscope. Stains can enhance and improve the visibility of the specimen. In addition, different stains have different affinities for various organelles and macromolecules."*

### 3.1. Material and methods

Cross sections were prepared from fresh roots of *A. rusticana* and *A. macrocarpa* by free hand technique. The sections were subjected to staining (i.e. toluidine blue, KI/iodine solution, and Sudan III) in order to enhance and improve the visibility of the specimen under the microscopic investigation. The sections were placed on a clean slide and flooded with aqueous solution of 0.2% toluidine blue for 5 min. The staining solution was then gently removed by using filter paper and the sections were repeatedly washed with water to get rid of unnecessary stain solution. The IKI solution was prepared by dissolving 1g of KI in 100 mL of water, followed by adding 1 g of iodine. The solutions was kept in a dark glass bottle and closed tightly to avoid the light and air. Each section was stained with 1 drop of IKI solution for few minutes before applying the cover glass. Sudan III solution was prepared by dissolving 0.01 g Sudan III in 5ml alcohol solution following by addition of 5 ml glycerin.

### 3.2. Results and discussion

As a polychromatic dye, toluidine blue reacts with different chemical components of the cells in different way and results in multi-colored specimen, which reveal the information on the structure of the cells. The cross sections of *A. rusticana* and *A. macrocarpa* roots showed the similarity of the two species,

i.e. the narrow core of primary xylem is surrounded by an extensive but mainly parenchymatous secondary xylem in which a few tracheary elements occur (figure 23). The well-defined vascular cambium also produces centrifugally a largely parenchymatous secondary phloem. IKI stained cross sections revealed starch present in cross sections based on the reaction of iodine and the center of the helical starch molecules, giving the dark blue color (longer molecules) or more red color (shorter molecules; figure 24). The oil content in the cross sections of *A. rusticana* and *A. macrocarpa* were investigated by exploiting the fat-soluble property of Sudan III stain, which is physically attracted to hydrophobic structures (figure 25, 26). These cross-sections showed the similarity in structure of fresh roots from both species – *A. rusticana* and its relative, *A. macrocarpa*.

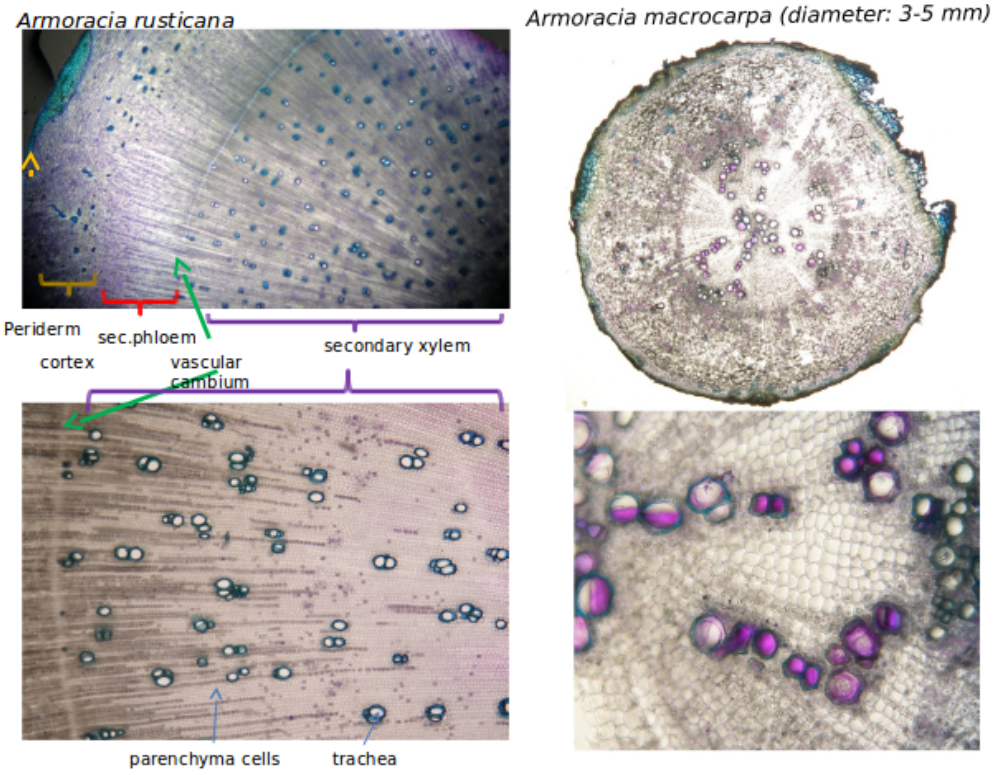


**Figure 23.** Cross sections of the fleshy roots of *Armoracia rusticana* and *Armoracia macrocarpa* stained with toluidine blue solution. The images were viewed with Olympus Provis AX70/A microscope.

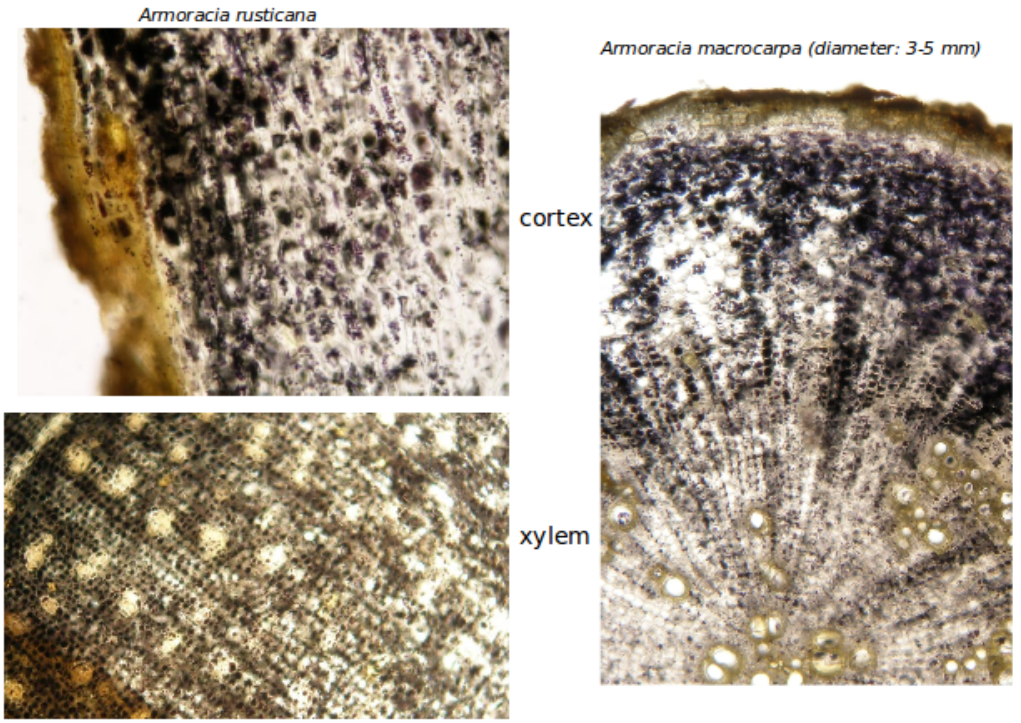



**Figure 24.** Cross sections of the fleshy roots of *Armoracia rusticana* and A*rmoracia macrocarpa* stained with IKI solution. The images were viewed with Olympus Provis AX70/A microscope.

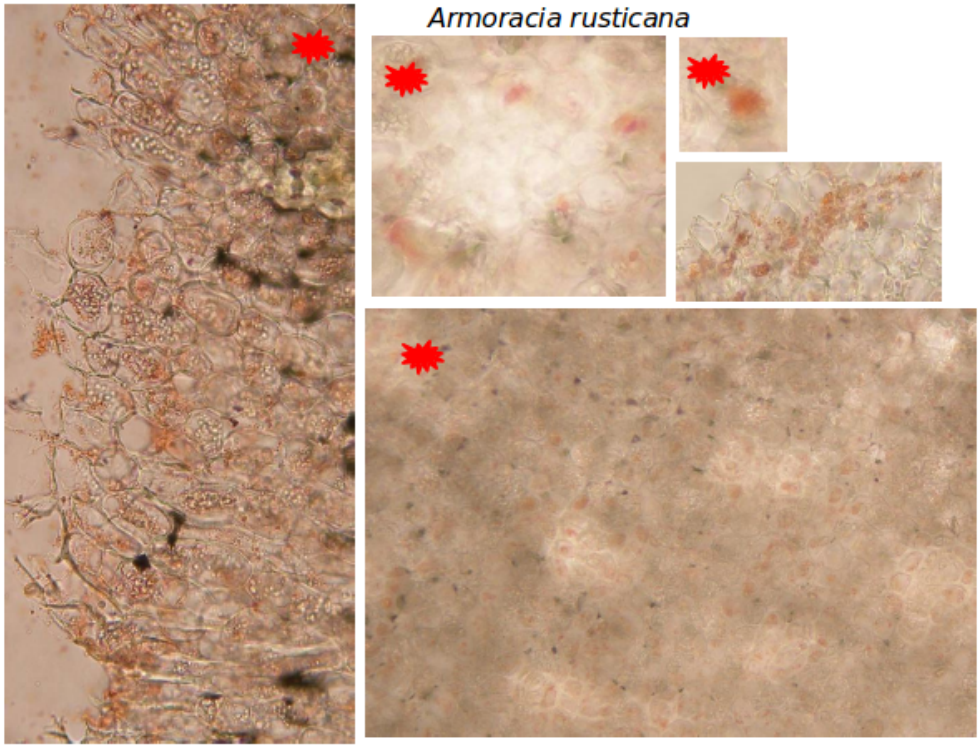



**Figure 25.** Cross section of the fleshy roots of *Armoracia rusticana* stained with Sudan III solution. The images were viewed with Olympus Provis AX70/A microscope.

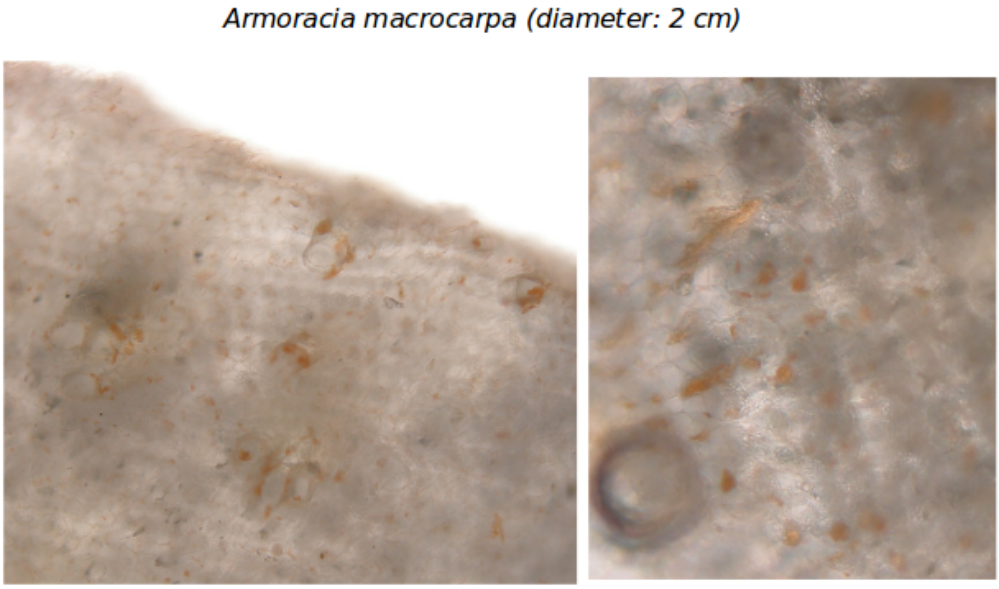



**Figure 26.** Cross section of the fleshy roots of Armoracia macrocarpa stained with Sudan III solution. The images were viewed with Olympus Provis AX70/A microscope.

## 4. THE GEL ELECTROPHORESIS STUDIES

Gel electrophoresis is a flexible method for separation and analysis of proteins, nucleic acids and other charged molecules. In electrophoresis, charged molecules are traveled through a porous gel by an applied electric field generated in a buffer which permeates the gel, and are separated based on their different electrophoretic mobilities. Variations in the gel and buffer make it possible to separate molecules not only based on their charges, but also on their molecular weight, isoelectric point and bio-specific affinity. The technique is fast, convenient, and inexpensive, and is used both as an analytical method and as a preparative procedure in the final stages of purification.

## 4.1. Materials and method

Roots and leaves of *A. rusticana* and A. *macrocarpa* were grind by commercial electric mixer. The material (approx. 1 g) was then transferred to 2 ml Eppendorf tubes, followed by the addition of 1mL buffer ($NaH_2PO_4/Na_2HPO_4$ 20 mM, 4°C, pH 6.55, VWR International Ltd.). The mixtures were aggressively mixed and centrifuged at 13000 rpm for 30 min using Heraeus Biofuge in order to obtain the supernatant. The protein content of the supernatant was assayed by the method of Bradford. 40 µg protein was loaded into each well of native 75% polyacrylamide gels. Electrophoresis was performed at 4°C. For myrosinase activity study, the gel was washed with distilled water and stained with solution (pH 8) containing 0.25 mL 20 mM $KH_2PO_4/K_2HPO_4$, 0.05 ml (169 mg/10 ml) Ascorbic acid, 0.5 mL 0.1% dye solution (methyl red), 1.25 (10 mg/mL) sinigrin and 2.95 mL water for 1-2 min. The myrosinase isoenzymes' band intensity were evaluated by ImageJ® and CP Atlas version 1.01 software. The data was graphically presented by Sigma plot 11.0 and Libreoffice Calc software. The activity of peroxidase was investigated using spectrophotometry (SHIMADZU, UV-1601). Each sample for spectrometry contains 970 µL 50mM $KH_2PO_4/K_2HPO_4$, 5 µL 3%$H_2O_2$, 20 µL 1 M pirogallol and 5 µL peroxidase enzyme from the supernatant.

## 4.2. Results and discussions

*Peroxidase activity* – Data obtained from spectrometry measurement (figure 27) compared the peroxidase activity in leaves, young root and old root from *A. rusticana* and *A. macrocarpa* collected in july. In *A. macrocarpa*, the highest activity was observed in the old root (801.05 ± 31.04 ΔOD/min/mg protein),

followed by the young root (412.6 ± 42.4 ΔOD/min/mg protein) and the least activity was in the leave (55.75 ± 1.43 ΔOD/min/mg protein). In *A. rusticana*, the same order of peroxidase activities were measured, i.e. the highest activity was recored in the old root (671.25 ± 31.5 ΔOD/min/mg protein), followed by the young root (206.95 ± 1.2 ΔOD/min/mg protein) and the least activities was measured in the leave (90.6 ± 2.4 ΔOD/min/mg protein). According to spectrometry results, the peroxidase activity in *A. macrocarpa* was higher compared to that in *A. rusticana*. Different results were recorded in other measurements with roots and leaves of both species collected in octorber, in which peroxidase activity was higher in *A. rusticana* (352.8 ± 1.4 ΔOD/min/mg protein, 16.4 ± 0.01ΔOD/min/mg protein in root and leaf, respectively) compared to that of *A. macrocarpa* (232.9 ± 0.57 ΔOD/min/mg protein, 27.72 ± 0.18 ΔOD/min/mg protein in root and leaf, respectively). The different results in these studies could be related to age and time of harvesting of roots.

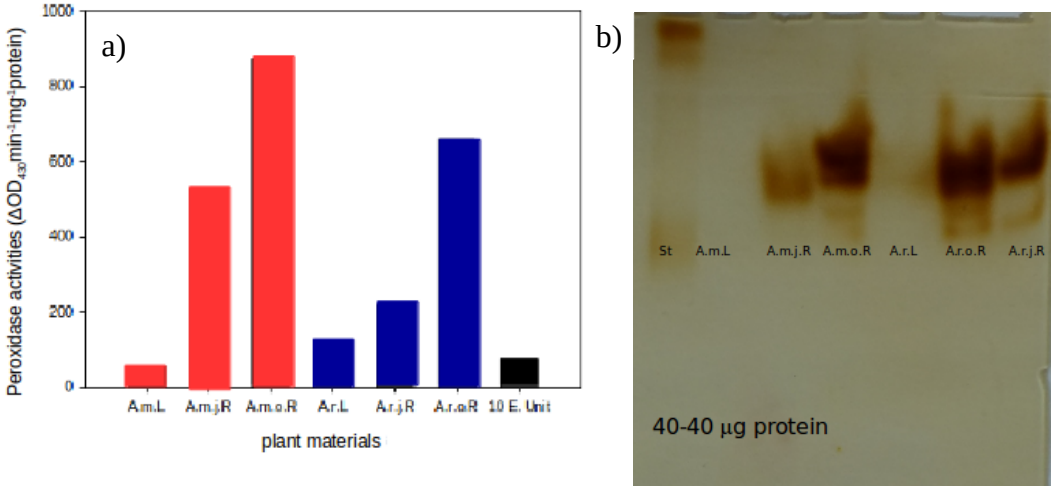



**Figure 27.** a) Peroxidase activities measured by spectrophotometry. (A.m: A*rmoracia macrocarpa*, A.r: *Armoracia rusticana*, L: Leaf, j.R: young root, o.R: old root). b) Peroxidase (E.C. 1.11.1.7) activity was visible due to dark red-colored purpurogallin bands showed on the gel. Picture was taken by

Olympus 4040 camera.

*Myrosinase activity* – The data on myrosinase activity was calculated based on the band intensities on the PEG gel (pixel per area – ppa). In *A. rusticana*, myrosinase activities in the young root and leave were similar (1378.67 ± 113.2 ppa and 1342 ± 49.66ppa). The highest activity was measured in the old root (1881 ± 110ppa). In case of *A. macrocarpa*, the enzymatic activity was higher in the roots (1628 ± 31.32ppa), in which the old root has the higher activity, similarly to the case of *A. rusticana*. Comparing 2 species, myrosinase activity was overall higher in *A. rusticana* compared to its relative. The highest myrosinase activity measured in *A. macrocarpa* old roots was similar to the activity measured in leave and young roots of *A. rusticana* (figure 28).

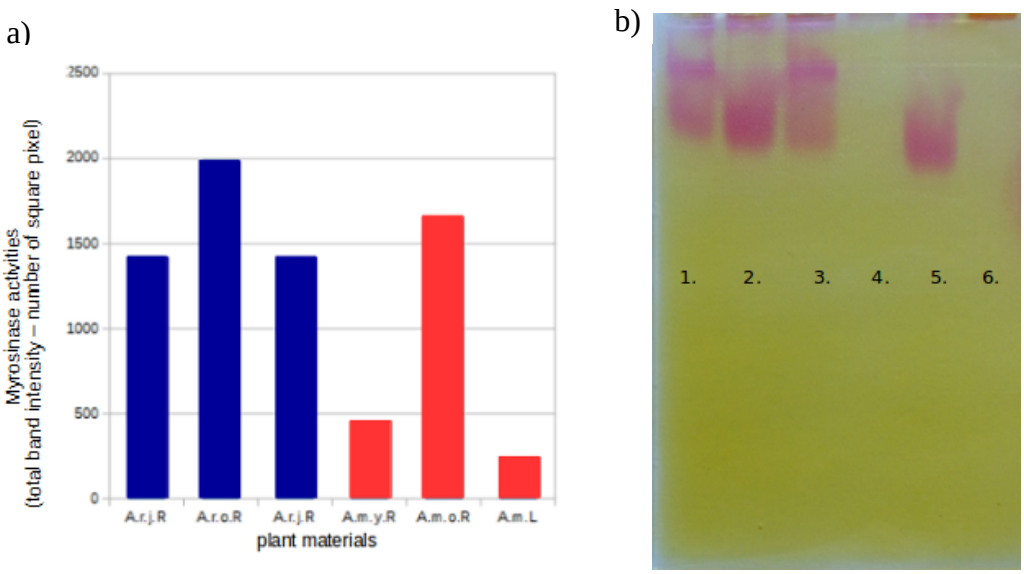



**Figure 28.** a) Gel electrophoresis study on the myrosinase activity in *A. rusticana* and its relative, *A. macrocarpa*. (1: *A. rusticana* young root, 2: *A. rusticana* old root, 3: *A. rusticana* leaf, 4: *A. macrocarpa* young root, 5: *A.*

*macrocarpa* old root, 6: *A. macrocarpa* leaf). b) Myrosinase activity measured by band intensities on gel. (A.r: *Armoracia rusticana*, A.m: *Armoracia macrocarpa*, L: leaf, y.R: young root, o.R: old root). Picture was taken by Olympus 4040 camera.

## Chapter V

## Summary

The first part of this study came up with a new technology to extracting high yield essential oil from fresh horseradish roots. With this method, for 15 kg of fresh horseradish root, 12ml of essential oil can be extracted (the yield of 0.08%). Together with the stable and high yield, the ease of use and its simplicity make sure that the new method is suitable for the horseradish essential oil production. Investigation on the quality of horseradish essential oil by gas chromatography and mass spectrometry showed the presences in high amount of 2 main substances: allyl isothiocyanate and 2-phenethyl isothiocyanate. The other three identified isothiocyanates are sec-butyl-, 3-butenyl- and 4-pentenyl isothiocyanate. Investigation on the completion of the extraction (no glucosinolate / isothiocyanate found in the post-distilled mush) and the “trapped” content of isothiocyanate (5 ppm) in watery extract by gas chromatography and mass spectrometry confirmed that the new method is recommended for large scaled horseradish oil production.

The second part of the study focuses on the development of new capillary electrophoresis (CE-MECK) method for simultaneous quantification of glucosinolates and isothiocyanates. The assay in short-end injection mode enables myrosinase quantification as well as glucosinolate' aglycon to isothiocyanate conversion rate estimation. The method uses sinigrin or gluconasturtiin as substrate, the main products of interest are derivatized to a more sensitively detectable dithiocarbamate product. It can also be a good alternative to established methods to quantify myrosinase activity from raw plant materials and similar matrices, as well as characterization of soluble

myrosinase enzymes, with respect to, for example, substrate specificity and pH optima. The method combines many advantages of frequently used methods: the specificity of chromatographic separations and the simplicity, low cost and time demand that is the property of the spectrophotometric assays.

The final part of this study compares the anatomical structure, glucosinolate profile and the enzymatic activities in both *A. rusticana* and *A. macrocarpa*. Study on cross sections stained with toluidine blue solution, IKI solution and Sudan III from *A. rusticana* and *A. macrocarpa* showed the similarity in anatomical structures of the root of both species. Study on glucosinolate profile in both species by liquid chromatography and mass spectrometry showed that there are 6 identified glucosinolates in *A. rusticana* and 16 glucosinolates were tentatively identified in *A. macrocarpa*. Gluconasturtiin, glucobrassicin, glucocochlearin, glucoconringianin and glucoibarin are the five glucosinolates found in both species. The absence of sinigrin (parent glucosinolate of allyl isothiocyanate) can be the possible explanation for the difference in the taste and smell of these plants.

The activities of peroxidase enzyme were recorded higher in *A. rusticana* compared to *A. macrocarpa* studied by spectrophotometry. However, the opposite results were also recorded, suggesting that peroxidase activity of both species heavily depends on the age and the time of harvesting of the roots. The old root had the highest activity compared to the young root and the leaf, where the lowest peroxidase activity was measured. The gel electrophoresis study on myrosinase enzyme activity showed higher activity in *A. rusticana* compared to *A. macrocarpa*. In *A. rusticana*, the old root had higher myrosinase activity than the young root and leaf, which had similar myrosinase activity. In *A.*

*macrocarpa*, the highest myrosinase activity was measured in the old root, then the young root and at last the leaf. The myrosinase activity in *A. rusticana* old root was higher than that of *A. macrocarpa* (20%), which was similar to the young root and leaf of *A. rusticana*.

List of new scientific results:

- Development of medium-scaled oil extraction method from fresh horseradish roots.
- Characterization of the main substances of horseradish oil produced in the crop provided by KELET Product Zrt.
- Development of capillary electrophoresis method for determination of glucosinolates and isothiocyanates from plant and food matrices.
- Characterization of myrosinase activity and release of allyl isothiocyanate of different vegetables.
- Comparative analysis of anatomical structure, glucosinolate profile and enzymatic activities of *A. rusticana* and *A. macrocarpa*.

## Chapter VI

## Acknowledgment

Firstly, I would like to express my sincere gratitude to my advisor Dr. Vasas Gábor and Dr. Sándor Gonda for the continuous support of my Ph.D study and related research, for their patiences, motivations, and immense knowledges. Their guidances helped me in all the time of research and writing of this thesis.

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## Chapter VII

## Bibliography

A. Depree, J., T. M. Howard, and G. P. Savage. 1998. “Flavour and Pharmaceutical Properties of the Volatile Sulphur Compounds of Wasabi (Wasabia Japonica).” *Food Research International* 31 (5): 329–37. doi:10.1016/S0963-9969(98)00105-7.

Agneta, Rosa, Anna Rita Rivelli, Emanuela Ventrella, Filomena Lelario, Giulio Sarli, and Sabino Aurelio Bufo. 2012. “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 Dissociation.” *Journal of Agricultural and Food Chemistry* 60 (30): 7474–82. doi:10.1021/jf301294h.

Bjergegaard, Charlotte, Peter Møller, Hilmer Sørensen, Jens Christian Sørensen, and Susanne Sørensen. 1999. “Micellar Electrokinetic Capillary Chromatography of Thiocarbamoyl Derivatives Produced in Reactions between Isothiocyanates and Amino Acids.” *Journal of Chromatography A* 836 (1): 115–27. doi:10.1016/S0021-9673(99)00109-0.

Björkman, Maria, Ingeborg Klingen, Andrew N. E. Birch, Atle M. Bones, Toby J. A. Bruce, Tor J. Johansen, Richard Meadow, et al. 2011. “Phytochemicals of Brassicaceae in Plant Protection and Human Health – Influences of Climate, Environment and Agronomic Practice.” *Phytochemistry* 72 (7): 538–56. doi:10.1016/j.phytochem.2011.01.014.

Bones, Atle M., and John T. Rossiter. 2006. “The Enzymic and Chemically Induced Decomposition of Glucosinolates.” *Phytochemistry* 67 (11):

1053–67. doi:10.1016/j.phytochem.2006.02.024.

Bruno, Robert D., and Vincent C.O. Njar. 2007. “Targeting Cytochrome P450 Enzymes: A New Approach in Anti-Cancer Drug Development.” *Bioorganic & Medicinal Chemistry* 15 (15): 5047–60. doi:10.1016/j.bmc.2007.05.046.

Budnowski, Julia, Franziska S. Hanschen, Carsten Lehmann, Michael Haack, Regina Brigelius-Flohé, Lothar W. Kroh, Michael Blaut, Sascha Rohn, and Laura Hanske. 2013. “A Derivatization Method for the Simultaneous Detection of Glucosinolates and Isothiocyanates in Biological Samples.” *Analytical Biochemistry* 441 (2): 199–207. doi:10.1016/j.ab.2013.07.002.

Cataldi, Tommaso R. I., Filomena Lelario, Donatella Orlando, and Sabino A. Bufo. 2010. “Collision-Induced Dissociation of the A + 2 Isotope Ion Facilitates Glucosinolates Structure Elucidation by Electrospray Ionization-Tandem Mass Spectrometry with a Linear Quadrupole Ion Trap.” *Analytical Chemistry* 82 (13): 5686–96. doi:10.1021/ac100703w.

Chen, Yi-Rong, Wenfu Wang, A.-N. Tony Kong, and Tse-Hua Tan. 1998. “Molecular Mechanisms of c-Jun N-Terminal Kinase-Mediated Apoptosis Induced by Anticarcinogenic Isothiocyanates.” *Journal of Biological Chemistry* 273 (3): 1769–75. doi:10.1074/jbc.273.3.1769.

Chiao, J. W., F.-L. Chung, R. Kancherla, T. Ahmed, A. Mittelman, and C. C. Conaway. 2002. “Sulforaphane and Its Metabolite Mediate Growth Arrest and Apoptosis in Human Prostate Cancer Cells.” *International Journal of Oncology* 20 (3): 631–36.

Clarke, John D., Roderick H. Dashwood, and Emily Ho. 2008. “Multi-Targeted Prevention of Cancer by Sulforaphane.” *Cancer Letters* 269 (2): 291–304. doi:10.1016/j.canlet.2008.04.018.

Dinkova-Kostova, Albena T., W. David Holtzclaw, Robert N. Cole, Ken Itoh, Nobunao Wakabayashi, Yasutake Katoh, Masayuki Yamamoto, and Paul Talalay. 2002. “Direct Evidence That Sulfhydryl Groups of Keap1 Are the Sensors Regulating Induction of Phase 2 Enzymes That Protect against Carcinogens and Oxidants.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (18): 11908–13. doi:10.1073/pnas.172398899.

“Extraction Technologies for Medicinal and Aromatic Plants.” 2016. Accessed July 21. http://www.academia.edu/7073099/Extraction_technologies_for_medicinal_and_aromatic_plants.

Fahey, Jed W., Amy T. Zalcmann, and Paul Talalay. 2001. “The Chemical Diversity and Distribution of Glucosinolates and Isothiocyanates among Plants.” *Phytochemistry* 56 (1): 5–51. doi:10.1016/S0031-9422(00)00316-2.

Fimognari, Carmela, Michael Nüsse, Rossano Cesari, Renato Iori, Giorgio Cantelli-Forti, and Patrizia Hrelia. 2002. “Growth Inhibition, Cell-Cycle Arrest and Apoptosis in Human T-Cell Leukemia by the Isothiocyanate Sulforaphane.” *Carcinogenesis* 23 (4): 581–86.

“GC-MS: Principle, Technique and Its Application in Food Science.” 2016. Accessed July 21. http://scholar.googleusercontent.com/scholar?q=cache:v-cNkIkOWzgJ:scholar.google.com/+GC+-+MS:+Principle,+Technique+and+its++application+in+Food+Science&hl=no&as_sdt=0,5&as_vis=1.

Gerhäuser, Clarissa, Karin Klimo, Elke Heiss, Isabell Neumann, Amira Gamal-Eldeen, Jutta Knauft, Guang-Yaw Liu, Somkid Sitthimonchai, and Norbert Frank. 2003. “Mechanism-Based in Vitro Screening of Potential

Cancer Chemopreventive Agents.” *Mutation Research* 523–524 (March): 163–72.

Gonda, Sándor, Nhat Minh Nguyen, Gyula Batta, Gyöngyi Gyémánt, Csaba Máthé, and Gábor Vasas. 2013. “Determination of Phenylethanoid Glycosides and Iridoid Glycosides from Therapeutically Used Plantago Species by CE-MEKC.” *Electrophoresis* 34 (17): 2577–84. doi:10.1002/elps.201300121.

Hanschen, Franziska S., Evelyn Lamy, Monika Schreiner, and Sascha Rohn. 2014. “Reactivity and Stability of Glucosinolates and Their Breakdown Products in Foods.” *Angewandte Chemie International Edition* 53 (43): 11430–50. doi:10.1002/anie.201402639.

Hecht, S. S. 2000. “Inhibition of Carcinogenesis by Isothiocyanates.” *Drug Metabolism Reviews* 32 (3–4): 395–411. doi:10.1081/DMR-100102342.

Herzallah, Saqer, and Richard Holley. 2012. “Determination of Sinigrin, Sinalbin, Allyl- and Benzyl Isothiocyanates by RP-HPLC in Mustard Powder Extracts.” *LWT - Food Science and Technology* 47 (2): 293–99. doi:10.1016/j.lwt.2012.01.022.

Hwang, Eun-Sun, and Hyong Joo Lee. 2006. “Phenylethyl Isothiocyanate and Its N-Acetylcysteine Conjugate Suppress the Metastasis of SK-Hep1 Human Hepatoma Cells.” *The Journal of Nutritional Biochemistry* 17 (12): 837–46. doi:10.1016/j.jnutbio.2006.02.004.

Ina, Kazuo, Akihito Sano, Mikako Nobukuni, and Isao Kishima. 1981. “Volatile Components of Wasabi (Wasabi Japonica) and Horse Radish (Cocholeria Aroracia).” *Nippon Shokuhin Kogyo Gakkaishi* 28 (7): 365–70. doi:10.3136/nskkk1962.28.7_365.

Ippoushi, Katsunari, Hidekazu Itou, Keiko Azuma, and Hisao Higashio. 2002. “Effect of Naturally Occurring Organosulfur Compounds on Nitric

Oxide Production in Lipopolysaccharide-Activated Macrophages." *Life Sciences* 71 (4): 411–19. doi:10.1016/S0024-3205(02)01685-5.

Jeong, Woo-Sik, Young-Sam Keum, Chi Chen, Mohit R. Jain, Guoxiang Shen, Jung-Hwan Kim, Wenge Li, and Ah-Ng Tony Kong. 2005. "Differential Expression and Stability of Endogenous Nuclear Factor E2-Related Factor 2 (Nrf2) by Natural Chemopreventive Compounds in HepG2 Human Hepatoma Cells." *Journal of Biochemistry and Molecular Biology* 38 (2): 167–76.

Karcher, A., and Z. El Rassi. 1999. "Capillary Electrophoresis of Glucosinolates and Their Degradation Products." *Electrophoresis* 20 (15–16): 3181–89. doi:10.1002/(SICI)1522-2683(19991001)20:15/16<3181::AID-ELPS3181>3.0.CO;2-G.

KJZER, ANDERS. 1963. "Mass Spectra of Isothiocyanates." *Acta Chem. Scand* 17 (8).

Kleinwächter, Maik, and Dirk Selmar. 2004. "A Novel Approach for Reliable Activity Determination of Ascorbic Acid Depending Myrosinases." *Journal of Biochemical and Biophysical Methods* 59 (3): 253–65. doi:10.1016/j.jbbm.2004.03.005.

Li, Xian, and Mosbah M. Kushad. 2005. "Purification and Characterization of Myrosinase from Horseradish (Armoracia Rusticana) Roots." *Plant Physiology and Biochemistry* 43 (6): 503–11. doi:10.1016/j.plaphy.2005.03.015.

Luciano, Fernando B, and Richard A Holley. 2009. "Enzymatic Inhibition by Allyl Isothiocyanate and Factors Affecting Its Antimicrobial Action against Escherichia Coli O157:H7." *International Journal of Food Microbiology* 131 (2–3): 240–45. doi:10.1016/j.ijfoodmicro.2009.03.005.

Mohlenbrock, Robert H. 1980. *Flowering Plants: Willows to Mustards*. SIU Press.

Murata, M., N. Yamashita, S. Inoue, and S. Kawanishi. 2000. “Mechanism of Oxidative DNA Damage Induced by Carcinogenic Allyl Isothiocyanate.” *Free Radical Biology & Medicine* 28 (5): 797–805.

Nehmé, Reine, Hala Nehmé, Grégory Roux, Deimante Cerniauskaite, Philippe Morin, Patrick Rollin, and Arnaud Tatibouët. 2014. “Contactless Conductivity Detection for Screening Myrosinase Substrates by Capillary Electrophoresis.” *Analytica Chimica Acta* 807 (January): 153–58. doi:10.1016/j.aca.2013.11.012.

Nielsen, P. V., and R. Rios. 2000. “Inhibition of Fungal Growth on Bread by Volatile Components from Spices and Herbs, and the Possible Application in Active Packaging, with Special Emphasis on Mustard Essential Oil.” *International Journal of Food Microbiology* 60 (2–3): 219–29.

Oerlemans, Kirsten, Diane M. Barrett, Carme Bosch Suades, Ruud Verkerk, and Matthijs Dekker. 2006. “Thermal Degradation of Glucosinolates in Red Cabbage.” *Food Chemistry* 95 (1): 19–29. doi:10.1016/j.foodchem.2004.12.013.

Ohta, Yoshio, Kenichi Takatani, and Shunro Kawakishi. 1995. “Decomposition Rate of Allyl Isothiocyanate in Aqueous Solution.” *Bioscience, Biotechnology, and Biochemistry* 59 (1): 102–3. doi:10.1271/bbb.59.102.

Piekarska, Anna, Barbara Kusznierewicz, Magdalena Meller, Karol Dziedziul, Jacek Namieśnik, and Agnieszka Bartoszek. 2013. “Myrosinase Activity in Different Plant Samples; Optimisation of Measurement Conditions for Spectrophotometric and pH-Stat Methods.” *Industrial Crops and*

*Products* 50 (October): 58–67. doi:10.1016/j.indcrop.2013.06.048.

Sampliner, Danielle, and Allison Miller. 2009. “Ethnobotany of Horseradish (Armoracia Rusticana, Brassicaceae) and Its Wild Relatives (Armoracia Spp.): Reproductive Biology and Local Uses in Their Native Ranges.” *Economic Botany* 63 (3): 303–13. doi:10.1007/s12231-009-9088-1.

Shehata, Ashraf, Richard M. S. Mulwa, Mohammad Babadoost, Mark Uchanski, Margaret A. Norton, Robert Skirvin, and S. Alan Walters. 2009. “Horseradish: Botany, Horticulture, Breeding.” In *Horticultural Reviews*, edited by Jules Janick, 221–261. John Wiley & Sons, Inc. http://onlinelibrary.wiley.com/doi/10.1002/9780470593776.ch4/summary.

Shin, Il Shik, Hideki Masuda, and Kinae Naohide. 2004. “Bactericidal Activity of Wasabi (Wasabia Japonica) against Helicobacter Pylori.” *International Journal of Food Microbiology* 94 (3): 255–61. doi:10.1016/S0168-1605(03)00297-6.

Singh, Ajita V., Dong Xiao, Karen L. Lew, Rajiv Dhir, and Shivendra V. Singh. 2004. “Sulforaphane Induces Caspase-Mediated Apoptosis in Cultured PC-3 Human Prostate Cancer Cells and Retards Growth of PC-3 Xenografts in Vivo.” *Carcinogenesis* 25 (1): 83–90. doi:10.1093/carcin/bgg178.

Smith, Tracy K., Elizabeth K. Lund, Mary L. Parker, Rosemary G. Clarke, Ian T. Johnson, and Nr Ua. 2004. *Allyl Isothiocyanate Causes Mitotic Block, Loss of Cell Adhesion and Disrupted Cytoskeletal Structure in HT29 Cells.*

Song, Lijiang, John J. Morrison, Nigel P. Botting, and Paul J. Thornalley. 2005. “Analysis of Glucosinolates, Isothiocyanates, and Amine Degradation Products in Vegetable Extracts and Blood Plasma by LC-MS/MS.”

*Analytical Biochemistry* 347 (2): 234–43. doi:10.1016/j.ab.2005.09.040.

Srivastava, Sanjay K, Dong Xiao, Karen L Lew, Pamela Hershberger, Demetrius M Kokkinakis, Candace S Johnson, Donald L Trump, and Shivendra V Singh. 2003. “Allyl Isothiocyanate, a Constituent of Cruciferous Vegetables, Inhibits Growth of PC-3 Human Prostate Cancer Xenografts in Vivo.” *Carcinogenesis* 24 (10): 1665–70. doi:10.1093/carcin/bgg123.

Sultana, Tamanna (author), G. P. (author) Savage, D. L. (authorDNRE McNei, N. G. (author) Porter, and B. (author) Clark. 2003. “Comparison of Flavour Compounds in Wasabi and Horseradish.” http://agris.fao.org/agris-search/search.do?recordID=FI2016100219.

Tagliaro, F, G Manetto, F Crivellente, and F. P Smith. 1998. “A Brief Introduction to Capillary Electrophoresis.” *Forensic Science International* 92 (2–3): 75–88. doi:10.1016/S0379-0738(98)00010-3.

Tang, Li, and Yuesheng Zhang. 2004. “Dietary Isothiocyanates Inhibit the Growth of Human Bladder Carcinoma Cells.” *The Journal of Nutrition* 134 (8): 2004–10.

———. 2005. “Mitochondria Are the Primary Target in Isothiocyanate-Induced Apoptosis in Human Bladder Cancer Cells.” *Molecular Cancer Therapeutics* 4 (8): 1250–59. doi:10.1158/1535-7163.MCT-05-0041.

Tsao, Rong, Qing Yu, John Potter, and Mikio Chiba. 2002. “Direct and Simultaneous Analysis of Sinigrin and Allyl Isothiocyanate in Mustard Samples by High-Performance Liquid Chromatography.” *Journal of Agricultural and Food Chemistry* 50 (17): 4749–53.

Tunc, S., E. Chollet, P. Chalier, L. Preziosi-Belloy, and N. Gontard. 2007. “Combined Effect of Volatile Antimicrobial Agents on the Growth of Penicillium Notatum.” *International Journal of Food Microbiology* 113

(3): 263–70. doi:10.1016/j.ijfoodmicro.2006.07.004.

Valgimigli, Luca, and Renato Iori. 2009. “Antioxidant and pro-Oxidant Capacities of ITCs.” *Environmental and Molecular Mutagenesis* 50 (3): 222–37. doi:10.1002/em.20468.

Vastenhout, Kayla J., Ruthellen H. Tornberg, Amanda L. Johnson, Michael W. Amolins, and Jared R. Mays. 2014. “High-Performance Liquid Chromatography-Based Method to Evaluate Kinetics of Glucosinolate Hydrolysis by Sinapis Alba Myrosinase.” *Analytical Biochemistry* 465 (November): 105–13. doi:10.1016/j.ab.2014.07.017.

Veitch, Nigel C. 2004. “Horseradish Peroxidase: A Modern View of a Classic Enzyme.” *Phytochemistry* 65 (3): 249–59.

Wilkinson, A. P., M. J. Rhodes, and G. R. Fenwick. 1984. “Determination of Myrosinase (Thioglucoside Glucohydrolase) Activity by a Spectrophotometric Coupled Enzyme Assay.” *Analytical Biochemistry* 139 (2): 284–91.

Xiao, Dong, Sanjay K. Srivastava, Karen L. Lew, Yan Zeng, Pamela Hershberger, Candace S. Johnson, Donald L. Trump, and Shivendra V. Singh. 2003. “Allyl Isothiocyanate, a Constituent of Cruciferous Vegetables, Inhibits Proliferation of Human Prostate Cancer Cells by Causing G2/M Arrest and Inducing Apoptosis.” *Carcinogenesis* 24 (5): 891–97.

Xu, K, and P J Thornalley. 2001. “Signal Transduction Activated by the Cancer Chemopreventive Isothiocyanates: Cleavage of BID Protein, Tyrosine Phosphorylation and Activation of JNK.” *British Journal of Cancer* 84 (5): 670–73. doi:10.1054/bjoc.2000.1636.

Yu, E. Y., I. J. Pickering, G. N. George, and R. C. Prince. 2001. “In Situ Observation of the Generation of Isothiocyanates from Sinigrin in

Horseradish and Wasabi." *Biochimica Et Biophysica Acta* 1527 (3): 156–60.

Yu, R, S Mandlekar, K J Harvey, D S Ucker, and A N Kong. 1998. "Chemopreventive Isothiocyanates Induce Apoptosis and Caspase-3-like Protease Activity." *Cancer Research* 58 (3): 402–8.

Zhang, Yuesheng. 2004. "Cancer-Preventive Isothiocyanates: Measurement of Human Exposure and Mechanism of Action." *Mutation Research* 555 (1–2): 173–90. doi:10.1016/j.mrfmmm.2004.04.017.

———. 2010. "Allyl Isothiocyanate as a Cancer Chemopreventive Phytochemical." *Molecular Nutrition & Food Research* 54 (1): 127–35. doi:10.1002/mnfr.200900323.

Zhang, Yuesheng, Li Tang, and Veronica Gonzalez. 2003. "Selected Isothiocyanates Rapidly Induce Growth Inhibition of Cancer Cells." *Molecular Cancer Therapeutics* 2 (10): 1045–52.

Zhao, Dayun, Jian Tang, and Xiaolin Ding. 2007. "Analysis of Volatile Components during Potherb Mustard (Brassica Juncea, Coss.) Pickle Fermentation Using SPME–GC-MS." *LWT - Food Science and Technology* 40 (3): 439–47. doi:10.1016/j.lwt.2005.12.002.

Zi-Tao Jiang, Rong Li. 2006. "Pungent Components from Thioglucosides in Armoracia Rusticana Grown in China, Obtained by Enzymatic Hydrolysis." *Food Technology and Biotechnology* 44 (1).

Zsolnai, T. 1971. "[Antimicrobial effects of thiocyanates, isothiocyanates and potential isothiocyanate forming substances]." *Arzneimittel-Forschung* 21 (1): 121–27.

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**List of publications related to the dissertation**

Foreign language scientific articles in international journals (2)

1. Gonda, S., Kiss-Szikszai, A., Szűcs, Z., **Nguyen, M. N.**, Vasas, G.: Myrosinase Compatible Simultaneous Determination of Glucosinolates and Allyl Isothiocyanate by Capillary Electrophoresis Micellar Electrokinetic Chromatography (CE-MEKC).
*Phytochem. Anal. 27* (3-4), 191-198, 2016. ISSN: 0958-0344.
DOI: http://dx.doi.org/10.1002/pca.2615
IF: 2.497 (2015)

2. **Nguyen, M. N.**, Gonda, S., Vasas, G.: A Review on the Phytochemical Composition and Potential Medicinal Uses of Horseradish (Armoracia rusticana) Root.
*Food Rev. Int. 29* (3), 261-275, 2013. ISSN: 8755-9129.
DOI: http://dx.doi.org/10.1080/87559129.2013.790047
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## List of other publications

Foreign language scientific articles in international journals (2)

3. Bertóti, R., Vasas, G., Gonda, S., **Nguyen, M. N.**, Szőke, É., Jakab, Á., Pócsi, I., Emri, T.: Glutathione protects Candida albicans against horseradish volatile oil.
*J. Basic Microbiol.* *56* (10), 1071-1079, 2016. ISSN: 0233-111X.
DOI: http://dx.doi.org/10.1002/jobm.201600082
IF: 1.585 (2015)

4. Gonda, S., **Nguyen, M. N.**, Batta, G., Gyémánt, G., Máthé, C., Vasas, G.: Determination of phenylethanoid glycosides and iridoid glycosides from therapeutically used Plantago species by CE-MEKC.
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