Egyetemi doktori (PhD) értekezés tézisei

STUDY ON HORSERADISH (*Armoracia rusticana*) ESSENTIAL OIL AND COMPARISON WITH THE RELATED SPECIES – DEBRECENI HORSERADISH (*Armoracia macrocarpa*).

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Chapter 1
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 he relative species of *Armoacia 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 2
Essential oil distillation technology

1. Small scale distillation

1.1. Materials and sample preparation

Fresh horseradish main and lateral roots were provided by KELET PRODUCT Zrt, Hungary. 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. 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

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.

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, sun flower oil and steam heating mode. A fractional column was also introduced before the condenser to improve the process (fractional distillation).

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 (<90ºC) 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 explanation 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^{o}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.

2. Medium scaled distillation

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. 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 spiral condenser was used to sufficiently process large amount of steam.

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). 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). Good result was achieved when the setting temperature falls around 95ºC, i.e. better yield (0.05%) compared to other temperature settings (i.e. 90ºC – 0.02%, 92.5ºC – 0.03% and 97.5ºC – 0.03%). 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%). 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.

Chapter 3

Analytical studies

1. Gas chromatography and mass spectrometry (GC / MS) study on horseradish essential

1.1. Sample preparation and method

Agilent 7890A GC 5975C MS instrument was used 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 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 (1 µl). The water extract were obtained from liquid / liquid extraction 10 times diluted with acetone. The water extract (1 µl) was directly injected.

1.2. Result and discussions

1.2.1. ITC components of horseradish essential oil

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). The essential oil extracted (large-scaled extracting) from horseradish from Újléta, Hungary showed major amount of AITC and PEITC. Together, AITC and PEITC composed of 98% of total ITCs where AITC comprised a higher proportion (85%). The AITC content is 2331.57mg/kg fresh horseradish roots. In the case of lateral roots, AITC level is only 382.12mg/kg fresh roots.

2. Capillary electrophoresis study

2.1. 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. 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). Sinigrin was quantified at 230, gluconasturtiin at 210, ITC derivates at 275 nm.

2.2. Method

2.2.1. 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.

2.2.2. Derivatization study

Dithiocarbamate is the product of in-vial derivatization reaction of ITCs with mercaptoacetic acid (MAA). 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. 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.

2.2.3. 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.

2.2.4. 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.

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.

2.2.5. Myrosinase activity determination and AITC release study

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 same vegetable extracts were also assayed for myrosinase activity by the widely used pH stat assay. 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%.

2.3. Results

2.3.1.Optimization of separation

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.

2.3.2. Derivatization study

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. 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.

2.3.3. Validation

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.

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.

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. If desired, an enzyme containing sample subjected to study could be injected about every 4 minutes.

2.3.4. 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. 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%.

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. The

presented long end injection method separates the analytes of interest within 15 minutes from real matrices. The presented method is similar in speed to the fastest available CE methods for glucosinolates, an analysis time of 15-25 minutes is typical. However, the separation of isothiocyanate adducts usually requires more time. With the new method, separation of allyl isothiocyanate dithiocarbamate was also done within 15 minutes.

2.3.5. Study of myrosinase activity and allyl isothiocyanate release of vegetable extracts

The activities (expressed in μmol sinigrin decomposed per minute (U)) of tested vegetables are recorded. 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%).

Chapter 4

Comparative analysis of *Armoracia rusticana* and *Armoracia macrocarpa*

1. Liquid chromatography and mass spectrometry study on glucosinolates

1.1. 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.

1.2. 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. These glucosinolates include sinigrin, gluconasturtiin, glucobrassicin, glucoiberin, glucococchlearin, and glucoconringianin. 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, glucococchlearin, 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 glucococchlearin, glucoconringianin, glucosativin, glucoibarin, 4-hydroxyglucobrassicin, 5-hydroxyglucobrassicin, glucobrassicin, gluconasturtiin, 4-methoxyglucobrassicin, and glucoarabishirsutain. 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).

2. Anatomical studies

2.1. 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.

3.2. Results and discussion

The cross sections (toluidine blue) 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. The well-defined vascular cambium also produces centrifugally a largely parenchymatous secondary phloem. IKI stained cross sections revealed starch present in cross sections. 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. These cross-sections showed the similarity in structure of

fresh roots from both species – *A. rusticana* and its relative, *A. macrocarpa*.

3. The gel electrophoresis studies

3.1. 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 peroxidase activity study, the gel was stained for 30-60 min in 50 mM KH_2PO_4/K_2HPO_4 (VWR), 0.015% (v/v) hydrogen peroxide and 20 mM pyrogallol. The activity of peroxidase was also 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. 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.

3.2. Results and discussions

Peroxidase activity – Data obtained from spectrometry measurement 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*. However, the result from GE showed the otherwise, i.e. the enzymatic activity for peroxidase was higher 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 the age and the time of harvesting of roots.

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*.

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.

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A PhD értekezés alapjául szolgáló közlemények

Idegen nyelvű tudományos közlemények külföldi folyóiratban (2)

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További közlemények

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