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Parameters Influencing the Quality of Processed Horseradish

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Abstract

Horseradish, *Armoracia rusticana*, has been used since centuries in traditional cuisine. The state of Styria is the second largest cultivation area in Europe with an annual production of approximately 4 000 tones of horseradish.

The distinctive aroma of horseradish is caused by mustard oils, which mainly belong to the group of isothiocyanates. These isothiocyanates are stored in the cells in form of glucosinolates. When the cell is damaged, the spatial separation between precursor and enzyme is eliminated, which leads to the break down of the glucosinolates by the cleavage enzyme *myrosinase*. The spicy, pungent lachrymating aroma derives primarily from allyl isothiocyanate. For the consumer the major quality attribute of horseradish products is their pungency.

As Styrian producers of horseradish products want to establish and strengthen their products on the world wide market, they count on high quality products, whereupon a demand for analytical methods for the control of the quality of the products arises. Furthermore, analytical methods for product improvements are needed to evaluate the parameters influencing the pungency of horseradish products. This thesis was involved in the development of methods for the determination of the analytes sinigrin, *myrosinase*, allyl isothiocyanate and citric acid, as well as their implementation for determining the amount and distribution of these analytes in horseradish roots and grated horseradish products.

Kurzfassung

Kren oder Meerrettich, mit dem latainischen Namen *Armoracia rusticana*, wird seit Jahrhunderten in der traditionellen Küche verwendet. Die Steiermark ist Europas zweitgrößtes Anbaugebiet mit einer jährlichen Produktion von etwa 4 000 Tonnen Kren.

Das charakteristische Aroma von Kren wird durch Senföle verursacht, welche hauptsächlich zur Verbindungsklasse der Isothiocyanate gehören. Diese Isothiocyanate werden in den Zellen der Pflanzen in Form von Glucosinolaten gespeichert. Werden die Zellen verletzt so wird die räumliche Trennung zwischen den Vorstufen und dem Enzym aufgehoben, was zum Abbau der Glucosinolate in Gegenwart des Spaltungsenzymes *Myrosinase* führt. Das scharfe, stechend, tränenreizende Aroma wird hauptsächlich durch die Verbindung Allylisothiocyanat hervorgerufen. Für den Konsumenten ist die „schärfe“ des Krenproduktes das Hauptqualitätskriterium.

Steirische Produzenten setzen auf qualitativ hochwertige Produkte um sich auf dem weltweiten Markt mit ihren Krenprodukten zu positionieren und zu festigen. Damit verbunden entsteht eine Nachfrage nach Analysenmethoden um die Qualität der Produkte kontrollieren und nachweisen zu können. Des Weiteren werden zur Verbesserung der Produkte und zur Erhaltung der Konkurrenzfähigkeit derer neue analytische Methoden benötigt um verschiedene Parameter auf ihren Einfluss auf die Krenschärfe zu untersuchen. Diese Arbeit beschäftigt sich mit der Entwicklung von Methoden zur Bestimmung der Analysenparameter Sinigrin, *Myrosinase*, Allylisothiocyanat und Zitronensäure, wie auch deren Anwendung zur Bestimmung der Mengen und Verteilungen der Analyten in Krenwurzeln und geriebenen Krenprodukten.

AFFIDAVIT

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master‘s thesis.

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Contents

1 Introduction

Horseradish, *Armoracia rusticana*, is an old cultivar, which has been planted for centuries as a spice. Its aromatic quality is a spicy, pungent lachrymating aroma, which is cherished in combination with fish, meat and sausage products. One of the traditional cultivation areas in Europe is the state of Styria in Austria. With an annual production of 4 000 tones of horseradish it is the second largest cultivation area in Europe. In order to consolidate their position on the world wide market, Styrian factories focus on the production of high quality horseradish products. This leads to the demand for analytical methods which allow the control and the evidence of the quality of horseradish products. Furtheron, for the marketing it would be useful to introduce a quality parameter against which the quality can be bit. Moreover, there is a constant demand for the improvement of the products. Respective analytical methods are needed to study different parameters influencing the pungency of horseradish products.

The volatile constituents of horseradish [1–4] as well as their precursor glucosinolates [5–7] were already determined in different researches. However, besides these studies, the literature on the topic of horseradish is quite rare. Up to now, the studied influencing parameters on the pungency of horseradish has been limited to the genotype [7], the correlation of the glucosinolate content to the *myrosinase* activity [8] and the storage time and storage temperature of paste like horseradish products [9].

Armoracia rusticana is a member of the family of the *Brassicaceae*, to which also broccoli, cabbage and rape belong. Because of their large consumption and the anticarcinogenic properties of glucosinolates, there is plenty of literature on these crops. Based on the same biochemistry within this family, a literature research was performed gathering the influencing parameters on the glucosinolate content studied so far. Additionally, a summary of the methods developed for the determination of glucosinolates, isothiocyanates and citric acid was intended.

The pungency and therefore the quality of horseradish roots is determined by the volatile compound allyl isohtiocyanate. This compound is stored in respective plants in form of the glucosinolate sinigrin. If the plant is injured, the degradation enzyme *myrosinase* hydrolyses sinigrin to allyl isothiocyanate. In order to allow the control and the evidence of the quality of horseradish products and as basis for future studies for the evaluation of the parameters which influence the pungency, the aim of this thesis was to develop methods addressing these three analytes. In addition, a method for the determination of citric acid was developed, as citric acid decreases the enzyme activity and is added for the stabilization of horseradish products.

2 Theoretical Background

2.1 Horseradish

2.1.1 Scientific Classification

Figure 2.1: Horseradish blossoms (left), leaves (middle) and roots (right) [10]

Kingdom	Plantae
Order	Brassicales
Family	Brassicaceae
Tribus	Cardamineae
Genus	Armoracia
Species	Armoracia Rusticana [11]

2.1.2 Botanical Description

Horseradish is a persistent, perennial and herbaceous plant that forms characteristic green leaves and thick taproots. Above ground the growth height reaches between 50 to 120 cm and the roots can penetrate into the ground up to 60 cm. The leaves (see figure 2.1) are characterized by a glabrous, ovat-lanceolate (basal leaf) to lineal-lanceolate (upper leaf) form. The lamina is 10 to 60 cm long and 5 to 12 cm broad. The leaf margin of basal leaves is strongly crenated and slightly curled, whereby the upper leaves are almost smooth. The leaf area contains strongly protuberant nerves. As hibernating organ, horseradish plants form a perpendicular,

cylindrical, white succulent taproot (see figure 2.1). The length of the roots varies between 30 to 40 cm with a diameter of 4 to 6 cm. Roots are often multi-headed close to the leaf stems and knotted at the root-end with plenty of secondary roots and fibrils. On the outside the roots are erratic, striated and have a yellow-brownish color, inside the roots are white and fibrous.

In spring horseradish forms inflorescence (see figure 2.1), which blossom from mid May to July. The round scape with its botryoid blossom of 40 cm can reach heights of 1.20 m. A flower consists of numerous hermaphrodite blossoms with doubled perianth. The outer perianth is built from four elongated and blunt sepals with 2 to 4 mm length. The inner perianth consists of four white petals which are approximately twice the length of the sepals. There are six stamens with filaments and anther. Between the filaments, six glands are arranged, two laterally at the bottom of the short and one between each long filament and the chalice. The pistil is almost not perceptible. The stigma is hemispheric with a furrow on top. After successful pollination, pods of 4 to 6 mm length are formed with approximately 4 seeds. [12]

2.1.3 Cultivation

Horseradish is cultivated on soil, which allows easy rooting and an erect growth as well as easily machinable and deep layers of soil. Because horseradish produce only insufficient amounts of seeds, farmers use the secondary roots for cultivation. The secondary roots are collected in the year before and are stored in bunches in cool and moist sand till use. In spring the secondary roots are planted almost horizontally with specialized planters into the prepared field in distances within the row of 15 cm and between the rows of 70 cm. In order to prevent the formation of unwanted multi-headed roots, the young roots are uncovered at the beginning of June and all side shoots except the thickest one are removed. Further, the roots are uncovered once more to remove all side shoots and secondary roots except for the root crown. In wet years this procedure is repeated every month. This procedure is still done by hand and allows the harvest of thick, plain and uniform roots. When the leaves start to die back, the growth of the roots is at its maximum and the roots can be harvested. Because the roots are perennial, this can happen from autumn till spring. For the harvest specialized machines subvert the roots and bring them to the top of the soil, where they are collected by hand. Horseradish is easily storable which allows the transport over long distances and the distribution throughout the whole year. [13]

2.1.4 Cultivation Areas

Horseradish originates from eastern Europe [14] and is nowadays cultivated mainly in the United States of America and Europe. In the U.S., horseradish is grown on approximately 3.000 acres, which are located in California, New Jersey, Virginia, Illinois and Wisconsin [15]. Europe has

an annual production of around 25.000 tonnes of horseradish [16], whereby Hungary is the biggest producer with approximately 12 000 tonnes [16], followed by Austria with 4.000 tonnes [17] and Germany. Other small producers are France in the area of Elsass and South Africa.

The horseradish cultivation in Austria is strongly focused on the state of Styria with 325 acreas. Some small cultivation can also be found in Burgenland, lower Autsria and upper Austria with 1 to 2 acres each [17]. Within Styria the traditional cultivation areas are in the southerly and easterly located districts Hartberg-Fürstenfeld, Deutschlandsberg, Voitsberg, Leibnitz, Weiz, Graz-Umgebung and Südoststeiermark [13].

2.1.5 Culinary Use

Mustard oils, which derive from the break down of glucosinolates into isothiocyanates, are responsible for the distinctive aroma of horseradish. The aroma is dominated by a spicy, pungent lachrymating (nasal trigeminal pain reaction) olfactory impression. Horseradish found its popularity in previous times, where it was, besides pepper and mustard, the only hot spice in large areas of Europe. Especially in the cultivation areas, horseradish is still used in kitchen because of its characteristics. Either freshly grated or in a paste like form, horseradish is served to meat, fish and sausage products. Nowadays the assortment reaches from fresh roots over grated horseradish to different combinations of paste like products like horseradish cream sauce, apple sauce, lingonberry sauce, mustard or sweet mustard sauce [18]. If the roots are dried or cooked, the volatile mustard oils are evaporated and therefore their pungency is lost [19]. Furthermore *myrosinase* is deactivated at 65 °C [20].

2.1.6 Medicinal Use

Long before horseradish was used as a spice, it started its career as a medicinal plant. It had already been known in ancient Egypt and China and was discovered in Europe for its medicinal properties in the middle age. Horseradish was used as a treatment for jaundice, affections of the respiratory tract and scurvy. Also its bactericide, fungicide and virus inhibiting properties were recognized early, although a scientific proof was only just possible in the last decades. [21]

Today the effectiveness of horseradish against respiratory and urinary passage infections is well documented and can be explained by its antibacterial properties, which can be attributed to the presence of isothiocyanates. Also ointments of horseradish demonstrably show alleviation of rheumatism and muscle pain [22]. Besides these two well known fields of applications, recently a new field opened for the use of *Brassicaceae* including horseradish in cancer prevention.

Isothiocyanates, the volatile constituents of horseradish, have shown very interesting activities against several diseases like cardiovascular diseases, chronicdegenerative diseases including cancer, diabetes and neurodegeneration. This broad field of application as a drug strongly boosted the research in this area. At first one would think it is impossible that one substance can inhibit such a variety of diseases. But the pathogenic determinants and risk factors are common to many chronicdegenerative diseases. For example, chronic inflammation is a major driving force for the development of many chronic diseases including atherosclerosis, cancer and insulin resistance. Also neuroinflammation leads to neurodegenerative diseases like Parkinson.

The biological activity of isothiocyanates is related to the electrophilic carbon residue located in the isothiocyanat moiety, which is able to react with biological nucleophiles. It is thought that the modification of proteins is the key mechanism in the biological activity of isothiocyanates. On a biochemical level isothiocyanates lead to an induction of phase II enzymes (detoxification enzymes) and inhibition of phase I enzymes (activation enzymes) of the biotransformation system. In the biotransformation phase I enzymes introduce functional groups into exogenous substances, which are then coupled in the phase II system to endogenous, water soluble substrates, which allows the excretion via the kidneys. Using the example of cancer, inhibition of phase I enzymes like *cytochrom P450* lowers the possibility of the presence of cancer inducing substances. This is due to the fact that *cytochrom P450* enzymes show a very low substrate specificity and therefore a metabolic activation of benign substrates into carcinogens such as aflatoxins or nitrosamines can occur. In the case of phase II enzymes an activation of the NRF2 system takes place, which controls the expression of a great number of antioxidant genes. Important systems that are regulated by this set of genes are drug detoxifying enzymes as NAD(P)H oxidoreductase and glutathione-S-transferase, heme oxygenase, which is an antioxidant defense enzyme, and enzymes of the glutathione synthesis. Besides the modulation of the biotransformation system, glucosinolates and their break down products also regulate cancer cell development through stopping the cell cycle and facilitating apoptosis. At last, inflammation derived diseases are attenuated by the ability of isothiocaynates to regulate the expression of proinflammatory cytokins by the inhibition of NF-κB. [23, 24]

In order to allow a discussion about the chemopreventive properties of isothiocyanates we also have to take into account their genotoxic potential. The electophilic reactivity of some of the isothiocyanates allows them to form adducts with DNA. This induces chromosomal aberrations and gene mutations. Additionally the dose-response relationship of genotoxic substances has no threshold. However, doses used in genotoxicity studies are several orders of magnitude higher than levels of isothiocyanates found in our dietary intake. Therefore such toxicities are very unlikely to occure in humans. [23]

2.1.7 Nutritional Aspects

In table 2.1 the nutrition values of horseradish are depicted. With twice the amount of vitamin C compared to lemon, plenty of other vitamins and minerals, horseradish is a high value foodstuff. The high amount of vitamin C explains its potency to prevent scurvy. However the most important constituent in the sense of aroma and its antibacterial and anticarcinogenic properties are the mustard oils, which mainly belong to the group of isothiocyanates. These isothiocyanates are stored in the cells in form of glucosinolates. When the cell is damaged the spatial separation between precursor and enzyme is eliminated, which leads to the break down of the glucosinolates by the cleavage enzyme *myrosinase*.

The main constituent of the mustard oil is allyl isothiocyanate, which primarily determines the spicy, pungent lachrymating aroma. Allyl isothiocyanate makes up to 64 to 82 % of the overall volatile fraction [4]. Its precursor glucosinolate is called sinigrin and supplies the highest amount of the overall glucosinolate content with 74 to 95 % [8]. In the following sections the glucosinolate-*myrosinase*-isothiocyanate system will be discussed in more detail.

Table 2.1: Nutrition values of horseradish (prepared) [25]

Nutrition		per 100 g
Water	g	85.08
Energy	kcal	48
Protein	g	1.18
Total lipid (fat)	g	0.69
Carbohydrate	g	11.29
Fiber, total dietary	g	3.3
Sugars, total	g	7.99
Minerals		
Calcium, Ca	mg	56
Iron, Fe	mg	0.42
Magnesium, Mg	mg	27
Phosphorus, P	mg	31
Potassium, K	mg	246
Sodium, Na	mg	420
Zinc, Zn	mg	0.83
Vitamins		
Vitamin C, total ascorbic acid	mg	24.9
Thiamine	mg	0.008
Riboflavin	mg	0.024
Niacin	mg	0.386
Vitamin B-6	mg	0.073
Folate, DFE	µg	57
Vitamin B-12	µg	0
Vitamin A, RAE	µg	0
Vitamin A, IU	IU	2
Vitamin E (α-tocopherol)	mg	0.01
Vitamin D (D2 + D3)	µg	0
Vitamin D	IU	0
Vitamin K (phylloquinone)	µg	1.3
Lipids		
Fatty acids, total saturated	g	0.09
Fatty acids, total monounsaturated	g	0.13
Fatty acids, total polyunsaturated	g	0.339
Fatty acids, total trans	g	0
Cholesterol	mg	0
Other		
Caffeine	mg	0

2.2 Glucosinolates

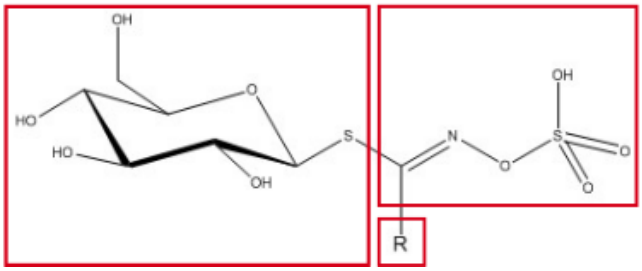


Figure 2.2: The scaffolding of glucosinolates consists of a β-D-glucopyranose moiety, which is linked via a sulfur atom to a (Z)-N-hydroxyiminosulfate ester, plus a variable R group. The high variety of the R groups leads to a large number of 120 known glucosinolates.

The storage form of the aroma active, antibacterial and anticarcinogenic isothiocyanates in *Brassicaceae* plants are the glucosinolates. Over 120 different glucosinolates are described in literature and they all share a common structure consisting of a (Z)-N-hydroxyiminosulfate ester, which is linked via a sulfur atom to a β-D-glucopyranose moiety and a variable R group. Each of these R groups originally derives from one out of eight amino acids [26]. Based on the type of modification to the R group and the precursor amino acid, the glucosinolates can be classified into aliphatic, aromatic and indole glucosinolates. Aliphatic glucosinolates derive from alanine, leucine, isoleucine, methionine and valine, in contrast aromatic glucosinolates derive from phenylalanine and tyrosine and indole glucosinolates from tryptophan [26]. The high number of known glucosinolates is related to the expensive modification of these precursor.

The biosynthesis of glucosinolates can be divided into three separate phases. In the first step most of the amino acids are elongated by one or more methylene residues (see figure 2.3). Second, independent of the extent of elongation, a metabolic reconfiguration takes place to form the core structure of glucosinolates (see figure 2.4). In the last step the R groups are subject to a set of transformations, including acylation, glucosylation, desaturation, O-methylation and hydroxylation. [27]

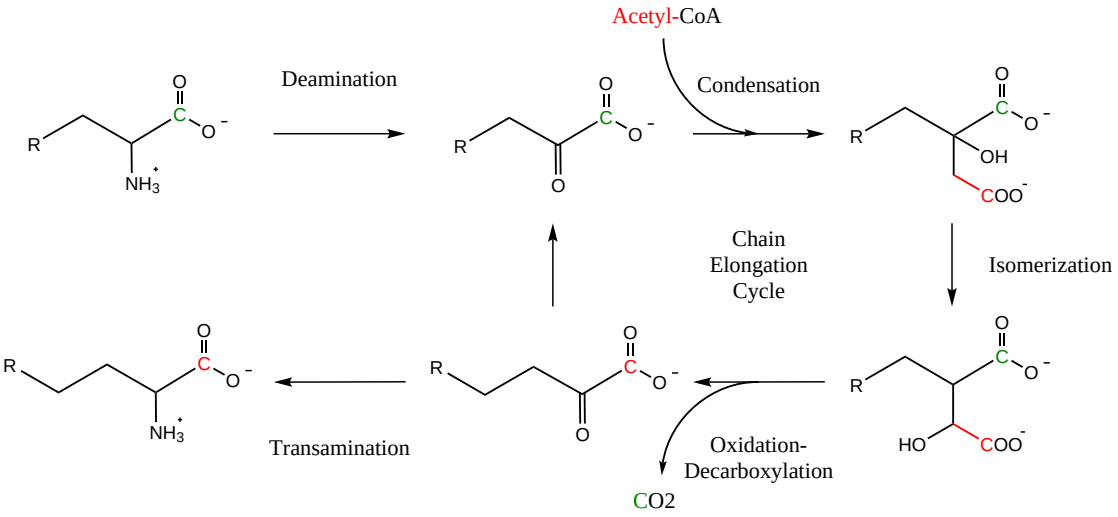


Figure 2.3: Illustrated is the chain elongation of amino acids of the first phase of glucosinolate biosynthesis. The chain elongation includes three principle steps: condensation with acetyl-CoA followed by an isomerization and an oxidative decarboxylation. The retained carbon atoms from acetyl-CoA are highlighted in red and the released carbon atoms from the amino acid carboxylate moiety are highlighted in green. [27]

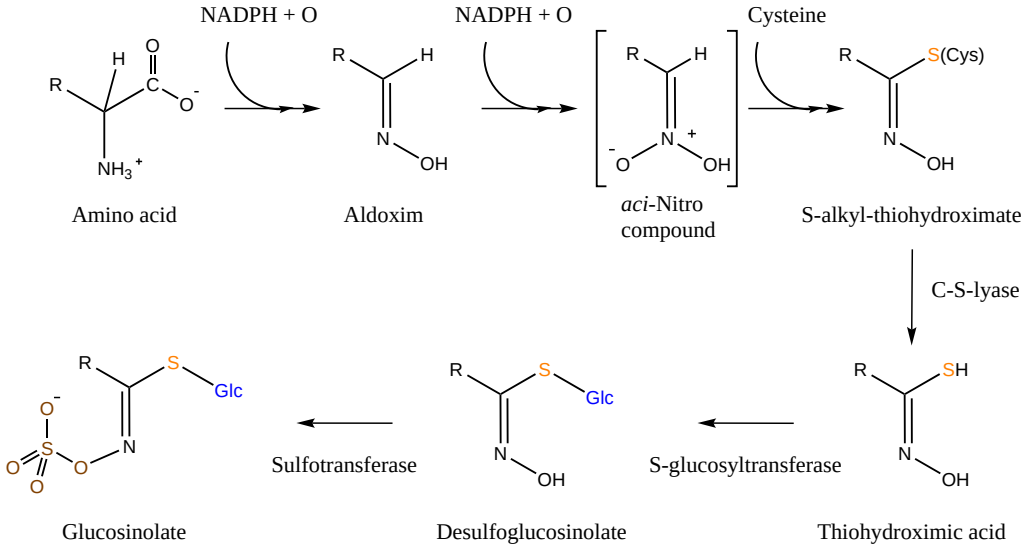


Figure 2.4: Illustrated is the core formation of the second phase of glucosinolate biosynthesis. The formation of the aldoxim is catalyzed by a cytochrom P450 of the family of CYP79. This is followed by the reaction to an *aci*-nitro compound in presence of the enzyme CYP83. In the next three steps the core of the glucosinolate is formed. First a S-alkyl-thiohydroximate is formed by the substitution of the hydrogen by a cystein. The catalyzing enzyme is still unknown, but it is suggested to be a glutathione-S-transferase type enzyme. Via C-S-lyase the cystein moiety is substracted, leaving a thiol moiety at the precursor. Now in presence of a S-glucosetransferase the β-D-glucopyranose moiety and the sulfate moiety, enabled by a sulfotransferase, are added. [27]

2.2.1 Biological Relevance

According to the great number of environmental factors, that show a strong influence on the glucosinolate pattern, like nutritional supply, light, fungal infection and insect damage, different theories were established to explain the potential role of glucosinolates in plants. The theories do not necessarily exclude each other. Following, the different theories are elucidated.

Nutritional supply *Brassicaceaes* have a high demand for sulfur. Therefore glucosinolates have been proposed to act like a sulfur storage pool, from which sulfur can be mobilized by hydrolysis. Further, glucosinolates also contain nitrogen, which suggests that the glucosinolate system acts as a sink for both nutrients. Different supplies of nitrogen and sulfur to *Brassica napus* demonstrated a strong influence on the amount of glucosinolates [28], whereby an increase in sulfur supply led to an increase of glucosinolates and vice verse. In contrast later studies have shown that sulfur is mainly stored as sulfate in the vegetative tissue [29] and that glucosinolates make up the smallest portion of the total sulfur content of *Brassica napus* [30]. Also a repression of most of the glucosinolate pathways under sulfur deficient conditions was found [31]. To sum up, their is a direct link between the sulfur supply and the glucosinolate amount, however it is unlikely that glucosinolates are the main source of recyclable sulfur [32].

Growth regulation The plant hormone indoleacetic acid (IAA) is an ubiquitous growth factor for plants. A possible link between IAA and glucosinolates is the indole class, which is supposed to hydrolyze to indole acetonitrile (IAN). IAN is then further subject to metabolisation with nitrilase to IAA [33]. Unfortunately the linkage between plant development and glucosinolate metabolism could not be completely solved and is still topic to further research.

Herbivore/insect defense According to the release of hydrolysis products of glucosinolates upon plant damage and their biological properties, it was long supposed that the crucial function of these compound classes is to repel herbivores and defend pathogens. Numerous studies demonstrated the feeding deterrence, growth inhibition and the high toxic potential of glucosinolate break down products to a wide variety of plant enemies, reaching from mammals to birds and insects and also including bacteria and fungi [27]. In all studies isothiocyanates turned out to be the responsible compound for these properties. Unfortunately, besides the ability to react with sulfurhydryl and amino groups of

proteins in vitro, the specific mechanism of isothiocyanates is still not resolved [34]. If a plant is damaged by insects, it responds with the accumulation of higher amounts of glucosinolates, which verifiably increases the resistance of these plants to subsequent attacks [35]. Besides unspecialized herbivores, there are herbivores which are adapted to glucosinolates and their break down products and use them as cues for oviposition and for feeding [36]. These herbivores developed different strategies to overcome the toxicity. For example *Plutella xylostella* uses an endogenous sulfatase to cleave the sulfate moiety off the glucosinolate core and therefore disables the formation of a *myrosinase*-glucosinolate complex [37]. Another strategy developed by *Pieris rapae* uses a protein factor to redirect the enzymatic cleavage from isothiocyanates towards the formation of nitriles, which exhibit a lower toxicity and are excreted with the feces [38]. The sequestering of glucosinolates is another strategy of specialized herbivores. In order to use sequestered glucosinolates for defense, these insects have their own endogenous *myrosinase* or rely on *myrosinase* in the guts of the enemies [39].

Pathogen defense The toxicity of glucosinolates metabolites against fungi and bacteria is extensively demonstrated for *in vitro* experiments [40][41], however *in vivo* studies, correlating pathogen resistance with glucosinolates, are only confirmed by a limited number of studies [42]. An interesting study showed greater yields of wheat grown on the same field after canola or Indian mustard compared to the control field, where wheat was grown in consecutive years [43]. This is in coincidents with a report that showed that the break down of *Brassica* tissue gives a high enough concentration of isothiocyanates in soil to sufficiently control pathogenic fungi [44]. Varying effects on different pathogens could be observed by different hydrolysis products [45]. If glucosinolates are exposed to soil from the decay of *Brassica* plants or root exudates, this alters the variety of the rhizosphere community. The present dominant soil fungal species near *Brassicaceae* have an increased tolerance to isothiocyanates and are different to species found elsewhere [46]. Moreover there are certain ecomycorrhizal species to whom hydrolysis products of indole glucosinolates act as growth stimulants [47].

2.2.2 Glucosinolates of Horseradish Roots

The family of *Brassicaceae* contains more than 3000 species and 350 genera, all of which are able to biosynthesise glucosinolates [48]. The concentration of glucosinolates in these plants is approximately 1 % of dry weight, but in different compartments also higher concentrations can occur, for example up to 10 % were found in seeds of some plants. From the known 120 glucosinolates, only a limited number is present in most species, namely not more than 12. Occasionally also numbers of 23 different glucosinolates have been found. Additionally, the distribution of glucosinolates varies quantitatively and qualitatively among leaves, stems, roots and seeds [26].

The total glucosinolate content of horseradish roots varies depending on the accession [7] from 1.8 mg/g dry weight to 13.8 mg/g dry weight. There are plenty of factors which influence the quantity and ratio of glucosinolates, as:

- Genotype
- Stage of maturity
- Point of harvest
- Soil conditions and fertilizer
- Mechanical wounding and feeding damages
- Phytohormones
- Climate
- Planting distance
- Plant compartment

which will be discussed in more detail in chapter 2.6. The three major glucosinolates in *Armoracia rusticana* are sinigrin, gluconasturtiin and glucobrassicin, all other glucosinolates make up of less than 0.1 % each of the overall glucosinolate content. Sinigrin is by far the most dominant glucosinolate and makes up to 74 - 95 % of the total glucosinolate content. In avarage fully developed roots contain approxiamety 83 % sinigrin, 11 % gluconasturiin and 1 % glucobrassicin [8]. A list of all glucosinolates found in *Armoracia rusticana* is given in table 2.2.

Table 2.2: Glucosinolates found in horseradish roots. [5–7]

Chemical name	Common name
Sulfur containing glucosinolates	
3-Hydroxy-5-(methylthio)pentyl	
3-(Methylsulfinyl)propyl	Glucoiberin
3-(Methylsulfonyl)propyl	Glucocheirolin
4-(Methylthio)butyl	Glucoerucin
2-(Methylthio)ethyl	Glucoviorylin
6-(Methylthio)hexyl	Glucolesquerellin
5-(Methylthio)pentyl	Glucoberteroin
3-(Methylthio)propyl	Glucoiberverin
Straight aliphatic glucosinolates	
n-Butyl	
Ethyl	Glucolepidiin
Methyl	Glucocapparin
n-Pentyl	
Branched aliphatic glucosinolates	
2-Methylbutyl	
3-Methylbutyl	
1-Methylethyl	Glucoputranjivin
Unsaturated aliphatic glucosinolates	
3-Butenyl	Gluconapin
5-Hexenyl	
2-Hydroxy-4-pentenyl	Gluconapoleiferin
4-Pentenyl	Glucobrassicanapin
2-Propenyl	Sinigrin⊛
Alcoholic aliphatic glucosinolates	
3-Hydroxybutyl	
2-Hydroxypentyl	
2-Hydroxypropyl	
Aryl aromatic glucosinolates	
Benzyl	Glucotropaeolin
2(R)-Hydroxy-2-phenylethyl	Glucobarbarin
2-Methoxybenzyl	
Phenyl	
4-Phenylbutyl	
2-Phenylethyl	Gluconasturtiin⊛
3-Phenylpropyl	
Indole glucosinolates	
Indol-3-ylmethyl	Glucobrassicin⊛

⊛ indicates the most dominant glucosinolates in *Armoracia rusticana*.

2.3 *Myrosinase*

The link between glucosinolates and the biologically active degradation products as isothiocyanates is the endogenous enzyme *myrosinase* (EC 3.2.3.1). *Myrosinase* is a member of the "Glycoside Hydrolase Family 1" [49], to which prokaryotic as well as eukaryotic enzymes like O-β-glucosidase and lactases belong. The three-dimensional structure and properties are closely related to those of more ubiquitous O-glucosidases [50]. As *myrosinases* have to evolve their activity in the external environment after tissue damage, the stability of the structure is promoted by the abundance of H-bonds, disulfide bridges and salt bridges. Additionally, *myrosinases* are strongly glucosinolated, making up to 20 % of their molecular masses. This in fact may protect the enzymes from inactivation by reactive degradation products and also enhances the protein stability [50]. The cleavage of thio-linked glucose residues is a property that so far has just been found with *myrosinases*, building the only group of β-thioglucosidases. This group is restricted to glucosinolates as substrates and cannot use O-glucoside or other S-glucoside substrates (in vitro) at all. However, within the different glucosinolates, *myrosinase* types can either hydrolyze multiple glucosinolate substrates [51] or are highly specific [52]. *Myrosinase* genes, so far known, are always encoded in a multigene family, which often have a distinct pattern of tissue- and organ-specific expression [53].

The mechanism of the hydrolysis of glucosinolates catalyzed by *myrosinase* is a two step reaction (see figure 2.5). In the active site two glutamic acid amino acids are involved in the catalysis. First the acidic/alkaline carboxylate moiety of the glutamic acid in the active center nucleophilically attacks the anomere carbon atom of the glucosinolate under the formation of a covalent glycosyl-enzyme-complex. In the second step, a water molecule attacks and releases the sugar moiety and at the same time regenerates the nucleophilic glutamic acid [54]. The products of the hydrolysis are a glucose molecule and an aglucon which is highly unstable and forms the corresponding isothiocyanate via Lossen-rearrangement.

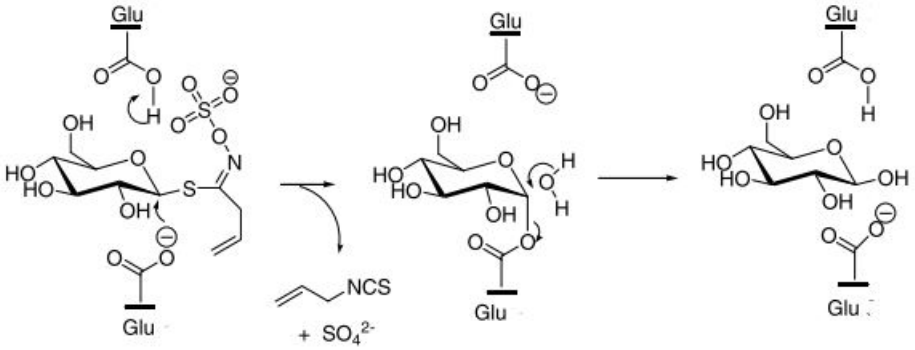


Figure 2.5: Catalysis of the glucosinolate hydrolysis by the β-thioglucosidase *myrosinase*. The substrate is Sinigrin which is hydrolyzed to glucose and the corresponding aglucon. This aglucon then spontaneously forms the allyl isothiocyanate in a Lossen-rearragement. [54]

2.4 Allyl Isothiocyanate

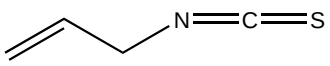
The aroma of horseradish is dominated by attributes like sulfury, bitter, mustard-, radish-, watercress like, pungent, sharp and lacrymatory. The flavor and odor active compounds responsible for these attributes turned out to be glucosinolates, e.g. sinigrin is a major source of bitterness, and their metabolites isothiocyanates, [55]. In table 2.3 the isothiocyanates found in horseradish roots are depicted. As can be ascertained, allyl isothiocyanate is the major isothiocyanate. Previous research reveals that allyl isothiocyanate makes up to 64 - 82 % of the overall volatile fraction [4], which is also in coincidence with the research shown in table 2.3. Besides allyl isothiocyanate only 2-phenetylethyl, *sec*-butyl and 3-butenyl isothiocyanate are found in higher amounts, all other isothiocyanates make up less then 0.4 % each of the overall volatile fraction. According to the low values of these isothiocyanates and their green, chemical and weak mustard like odor, the aroma of horseradish is strongly dominated by allyl isothiocyanate. Allyl isothiocyanate in its pure form has a bitter, strongly pungent, lacrymatory and mustard like odor, which gives horseradish its characteristic aroma.

Table 2.3: Depicted are the research results from 5 different research groups, showing the isothiocyanates found in horseradish roots, as well as their odor description.

Reference		[4]	[2]	[3]	[56]	[57]	[1, 2, 5, 58]
Unit		[mg/kg]	[mg/kg]	[%][1]	[%][1]	[%][1]	
Isopropyl	ITC	< 0.5		0.1			Chemical, weak mustard like
Allyl®	ITC	1570	966	78.4	74.5	53.5	Strongly pungent, mustard like, lacrymatory, bitter
n-Butyl	ITC		4.2	0.1			Sulfury, green, pungent
sec-Butyl	ITC	27			1.9	2.7	Chemical, weak mustard like
Isobutyl	ITC	0.6			0.2		Sweet, chemical
3-Butenyl	ITC	5.5	8.1	1.5	0.4		Green, pungent
Pentyl	ITC				0.02		-
4-Pentenyl	ITC	1.7	1			0.6	Green, pungent, acrid, fragrant
Propyl	ITC	1.6					Leaf, chemical, pungent
Phenyl	ITC			0.1			-
Benzyl	ITC	133	225	0.1	0.3		Strongly radish-like, pungent, strong, watercress aroma, tingling sensation
2-Phenylethyl®	ITC			9.4	9.5	39.2	Green
3-Methylbutyl	ITC				12.6		-
Sulphinyl pentyl	ITC		8.1				-
6-Methylsul -phinylhexyl	ITC		9				-
Total	ITC	1740.9	1231.0	92.1	99.4	96.0	

[1] % of the volatile fraction
® indicates the isothiocyanates of the most dominant glucosinolates in horseradish roots

2.4.1 Allyl Isothiocyanate as Quality Determining Parameter of Horseradish



Allyl isothiocyanate

Figure 2.6: Allyl isothiocyanate is the major constitutent of the volatile fraction of horseradish roots. As a pure substance it has a bitter, strongly pungent, lacrymatory and mustard like odor, which gives horseradish its characteristic aroma.

Generally, the acceptance of a product by the consumers is strongly linked to the flavor and taste of the product. The crucial parameters for the acceptance of *Brassicaceae* vegetables are their bitterness, their sulfury aroma and hotness. Studies have shown that the excessive presence of these three factors leads to a smaller acceptance by the consumers [59, 60]. However, in the case of horseradish, the bitterness and sulfury aroma play a minor role, as horseradish is used as a spice. Therefore the consumed amount and the consumption together with meat, fish or sausage products overlays the unwanted flavor and odors, or rather reduces them to a pleasant level. As a spice horseradish is appreciated by the consumers for its spicy, pungent lachrymating aroma. Therefore the major quality determining parameter besides the shape and color of the root and its flesh is the level of pungency. The more pungent a horseradish product is, the higher the quality of the product is from the perspective of the consumer. Therefore an analytical method that gives access to the amount of allyl isothiocyanate, which is responsible for the pungency, allows to determine the quality and acceptance of horseradish products. Furthermore, the expression of the quality in numbers may allow to control the quality routinely, compare products and to establish classes of pungency in the future.

2.4.2 The Nasal Trigeminal Pain Reaction

The pungent, lachrymatic pain reaction in presence of allyl isothiocyanate is a stimulation of the nasal trigeminal nerve system. Besides the olfactory and gustatory system, which allows us as our principal chemosensory systems to identify different odor and taste active compounds, we have a second system, the trigeminal system. This system is built up by the fifth cranial nerve (trigeminus), which innervates the skin covering our face, the mucous membranes of the nasal and oral cavities and the conjunctiva and cornea of the eyes. This system is responsible for the perception of temperature, touch and pain and furthermore plays a fundamental role in the perception of chemicals. Originally this system had a defensive and protective function as most of the stimulating substances are poisonous and irritants. The impressions of this system can be described as e.g. stinging, tingling, burning, itching, cold, warm and are accompanied by protective responses as tearing, respiratory depression, coughing, sneezing and salivation.

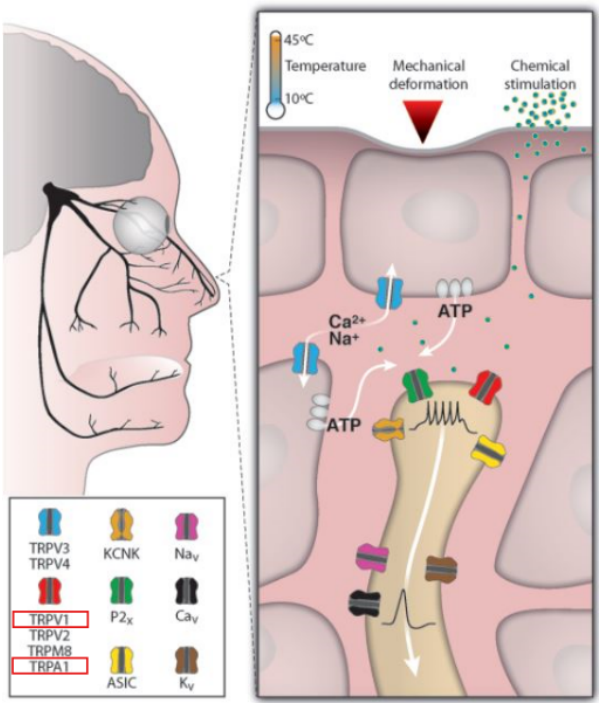


Figure 2.7: Sensory nerve terminals of the trigeminal system are directly stimulated by chemicals, temperature and touch. TRP... cationic channels (transient receptor potential ion channels), ASIC... cationic channels, KCNK... potassium selective channels, X_V... voltage gated channels [61]

However, over time humans have also developed a liking for the pungency and sharpness of many spices, as it is in the case of horseradish. Compared to the olfactory system, which has normal receptors (metabotropic mechanism), the trigeminal system has so called nozizeptors – free nerve endings. These free nerve endings contain ion channels that follow the mechanism of ionotropic transduction. In this case the change in ionic permeability and therefore the depolarization of the receptor is a consequence of direct gating of the ion channels by the chemical stimulus. For the transduction of the chemical signals from allyl isothiocyanate into a nerve impulse, transient receptor potential channels (TRP) are responsible. More precisely a stimulation of TRP receptor types A1 and V1 takes place, whereby the affinity to A1 is higher then to V1. These channels are porous for cations and have a binding site for different chemicals to home allyl isothiocyanate counts. So the pungent, lachrymatic pain reaction is a consequence of the stimulation of the specialized ion channels of the nerve endings of the trigeminal system. The response threshold of the trigeminal system is in an approximation to the olfactory threshold about 1 to 2 log units higher. [61]

2.5 The Glucosionlate-*Myrosinase*-Isothiocyanate System

The glucosinolate-*myrosinase*-isothiocyanate system is a very complex biochemical pathway that tends to maximize the survival chance of plants of the family of *Brassicaceae*. This system has to overcome different problems, like premature degradation of the glucosinolates by the degradation enzyme into the biologically active isothiocyanates. Competitive degradation pathways lead to different products, which on the other hand allow the repelling of different pests. How nature deals with these problems will be discussed in this chapter.

2.5.1 Spacial Separation of Glucosinolates from *Myrosinase*

Based on the theory of the "mustard oil bomb" [62], plants of the family of *Brassicaceae* are supposed to prevent the loss of glucosinolates in presence of the degradation enzyme *myrosinase* and therefore the ability to defend themselves against plant pests by a spatial separation of the precursor and enzyme. According to this theory, degradation of glucosinolates can only occur if the segregation is eliminated through damage of the tissue. This leads to three spatial distribution scenarios [63]:

1. Localization of the substrate and the enzyme in the same cell and same subcellular compartment but in an inactive form
2. Localization of the substrate and the enzyme in the same cell but different subcellular compartments
3. Localization of the substrate and the enzyme in different cells, either in different or in the same subcellular compartments

This theory is supported by the discovery that the degradation enzyme *myrosinase* is localized in myrosin-cells (M-cells) [64] and on the subcellular level that the enzyme is stored in so called myrosin-grains [65]. This early discovery in the 1890s could be confirmed by the use of antibodies and immunogold-EM in 1991 [66]. However the localization of the glucosinolates is still topic of research. Scenario one is supported by the fact that high concentrations of ascorbic acid lead to a decrease and low concentrations of ascorbic acid lead to an increase of the activity of *myrosinase* [67] (1976). According to the localization of ascorbic acid in vacuoles [6] it is suggested that the damage of the tissue leads to a decrease of the concentration of ascorbic acid and due to the dilution to an activation of *myrosinase* [63]. The second scenario with glucosinolates and *myrosinase* in different vacuoles but in the same cell seems to be unlikely, because the vacuoles of M-cells undergo fissions and fusions during maturing [68]. This would lead immediately to the degradation of the glucosinolates. A study from 1998 [69], with antibodies raised against sinigrin, showed with exception of M-cells a labeling for all other cells. A further study from 2000 [70] selectively analyzed the sulfur content of cells, whereby

one cell type showed a characteristic high content of sulfur (S-cells). Both studies seem to prove scenario three, but applying radio labeled glucosinolate precursors to seeds of *B. napus* showed radioactive labeling of specific cells. The pattern and number of these labeled cells is in accordance with the distribution of M-cells. To sum up, at the present time we know that *myrosinase* is stored in myrosinase-grains in so called M-cells and it is also confirmed that glucosinolates are also localized in vacuoles [71]. However, due to the contrary results the question whether the glucosinolates and the *myrosinase* are stored in vacuoles in the same cell or in different cells, remains open. So far the available data fit best the spatial separatio of scenario three.

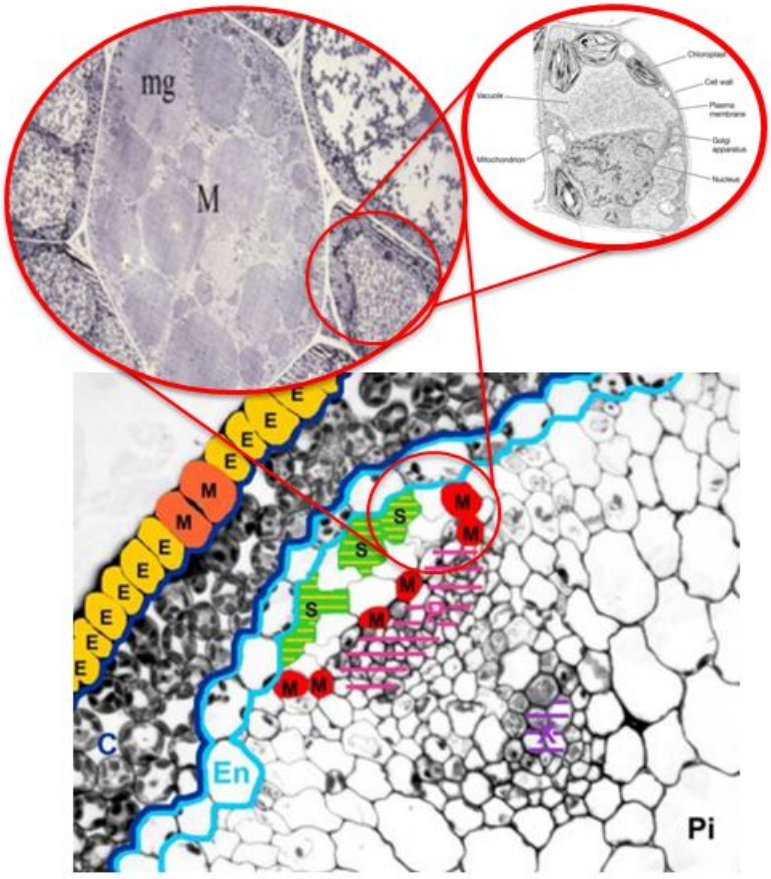


Figure 2.8: This figure represents the "mustard-oil-bomb". This theory assumes that the stability of glucosinolates in intact root tissue of *Brassicaceae* occurs due to a spatial separation of glucosinolates and *myrosinase*. Youngest research gives evidence to suggest that the spatial separation is given by the localization of *myrosinase* (red cells) and glucosinolates (green cells) in different cells. On the subcellular level both are stored in the vacuoles of the corresponding cells. The distribution of these cells is usually considered to be random, however a large proportion of these cells are localized in the second-outmost cell layer between the endodermis (light blue EN) and the phloem (pink P). Wounding of the tissue eliminates the segregation and isothiocyanates are formed. [72]

2.5.2 Enzymatic Degradation

The link between glucosinolates and their corresponding isothiocyanates is the degradation enzyme *myrosinase*. The mechanism will be discussed exemplary by the degradation of sinigrin to allyl isothiocyanate (see figure 2.9). In the first step *myrosinase* cleaves the thioglucose-bond of sinigrin, which leads to the elimination of the glucose moiety and to the formation of a thiohydroximate-O-sulfonate. This unstable intermediate is also called aglucone and in absence of any further influencing factors this aglucone will rearrange to the corresponding allyl isothiocyanate in a Losson-rearrangement. This rearrangement describes the migration of the side chain from the carbon at the oxime position to the nitrogen adjacent and is accompanied by the loss of the sulfate moiety. [73]

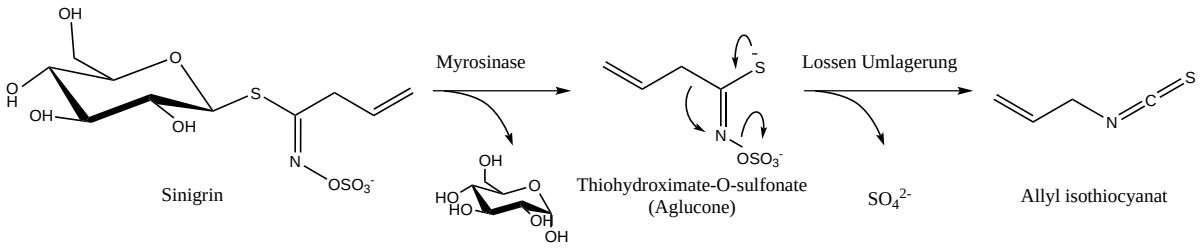


Figure 2.9: Depicted is the degradation pathway of sinigrin to allyl isothiocyanate. The black arrows on the unstable aglucone display the mechanism of the Lossen-rearrangement.

Plants possess a set of specifier proteins that allows the formation of organic thiocyanates, simple nitriles or epithionitriles. These specifier proteins are thought to act as enzymes on the aglucone to prevent the spontaneous formation of isothiocyanates by catalyzing competitive pathways. Which degradation product is formed depends on the type of the specifier protein and the chemical structure of the residue on the aglucone (see figure 2.10). Thiocyanate formation is catalyzed by the thiocyanate-forming protein (TFP), although this pathway is only known for allyl-, benzyl- and 4-methylthiobutylglucosinolate and occurs only in the presence of TFP. If the aglucone contains a terminal double bond, the formation of epithionitriles is catalyzed by the epithiospezifier protein (ESP) as well as by TFP. Nitrile-specifier proteins (NSP) promote the formation of simple nitriles, however in comparison to TFP and ESP, who also show a nitrile-specifier activity, NSP is restricted to the formation of nitriles and does not catalyze any other degradation pathway. Interestingly plants also possess an antagonist to the epithiospecifier-modifier protein (ESM), which probably blocks the activity of ESP and therefore favors the formation of isothiocyanates [74]. Finally, if the aglucone contains a side chain with a hydroxyl group at the C-2 position, the formed isothiocyanate is unstable and forms an oxazolidine-2-thione in a cyclization reaction. [75]

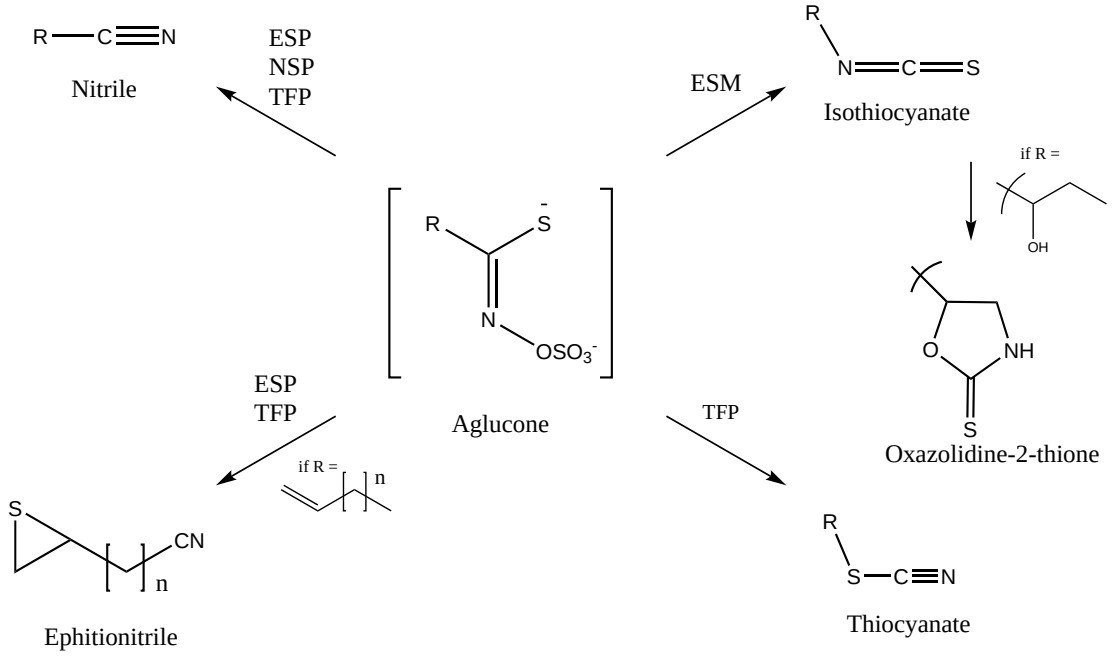


Figure 2.10: Plants possess a set of specifier enzymes which allow the formation of different degradation products. An explanation for this property is the response of plants against a broad variety of herbivores. (TFP thiocyanate-forming protein, ESP epithio-specifier protein, NSP nitrile-specifier protein, ESM epithiospecifier-modifier protein)

The advantage of this set of degradation products to plants has not completely been solved yet, though it is likely that the diverse spectrum of biologically active compounds enables the specific respons of plants to a broad range of enemies. The toxicity of isothiocyanates to general and specialized insects could be proven by various examples. Also for organic thiocyanates and simple nitriles examples have been found, in which they protect plants as toxins. But for epithionitriles no toxic effects have been noted so far. In general isothiocyanates show a higher toxicity than their competitive degradation products. An exception of this rule is allyl thiocyanate, which has a higher toxicity than allyl isothiocyanate. Based on several reasarches which studied the influence of plants with high amounts of ESP on different herbivores, one can say that the production of nitriles instead of isothiocyanates rather has an advantage for herbivores than for the plant itself. This conclusion is underpinned by the fact that larvae of specialized insects, e.g. *Pieris rapae*, use nitrile formation upon ingestion of glucosinolates and plant *myrosinase*, catalyzed by the gut nitrile-spezifier protein (GNSP), to overcome the toxicity of isothiocyanates. As nitriles might not act as toxins, it is thought that indirect defense responses may explain their protective effect on plants. One possibility is that nitriles act as pheromones and attract natural enemies of herbivores. Additionally, a reduction of isothiocyanates may reduce the ability of specialized insects to recognize *Brassicaceae* plants and therefore suppress oviposition. [76]

2.5.3 Influences on the Enzymatic Degradation

Besides the specifier enzymes, external factors as Fe^{2+}, pH value, humidity and temperature can influence the activity of the enzymes or directly favor one degradation pathway over the others. Nitriles and iosthiocyanates are in direct competition. The pH value during hydrolysis has a strong impact on the *myrosinase* activity. A low pH (< 5) favors the formation of nitriles due to the inhibition of the Losson-rearrangement, whereby a neutral to alkaline pH (> 5) leads to the formation of isothiocyanates [77]. Additionally, isothiocyanates are formed predominantly at higher temperatures and an elevated humidity and vice versa for nitriles [78]. Furthermore the presence of Fe^{2+} and thiole derivatives lead to an enhanced formation of nitriles [79]. Fe^{2+} is one of the factors which on the one side is able to induce the formation of nitriles directly and on the others side Fe^{2+} manipulates the activity of ESP and NSPs.

Table 2.4: There are different parameters which favor one degradation pathway over the other and determine which break down product is predominantly formed by the aglucone.

Isothiocyanate	Nitrile	Epithionitrile	Thiocyanate
ESM	NSP, ESP, TFP	ESP, TFP	TFP
absence of Fe^{2+}	presence of Fe^{2+}	presence of Fe^{2+}	
pH > 5	pH < 5		
higher temperature	lower temperature		
increased humidity	decreased humidity		
ascorbate			
hydrogensulfide			

When it comes to the activity of *myrosinase*, ascorbate plays a major role. In presence of ascorbate an acceleration of the turnover rate occurs. It was shown that this enhancement of the activity is caused by an alosteric effect [80], whereby ascorbate acts like a catalytic base [81] (see figure 2.11). But the regime is not linear, above a distinct concentration level a decrease of the activity takes place [67].

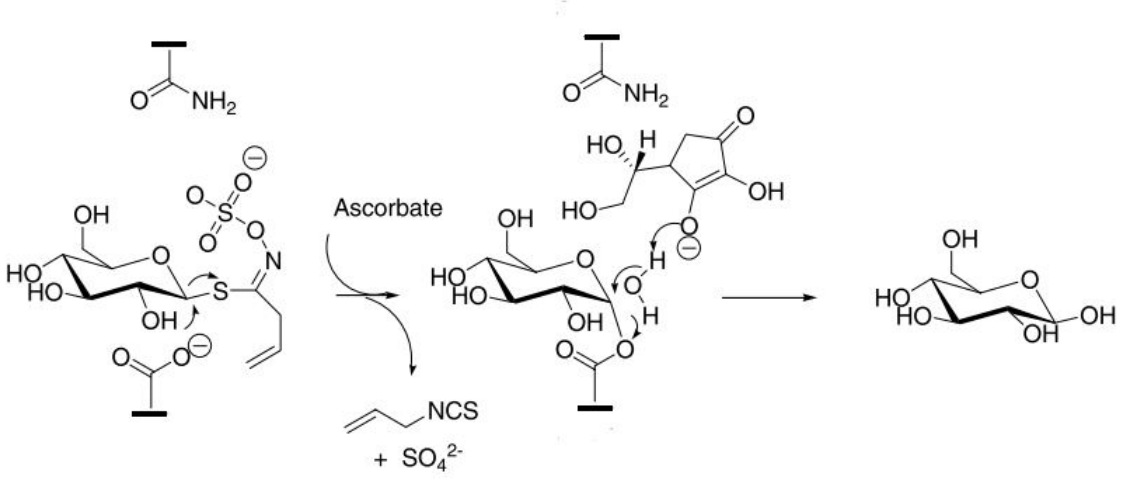


Figure 2.11: After the glucose moiety is eliminated from the aglucon, ascorbate accelerates the hydrolysis of the glucose residue bound to the enzyme by "activation" of water [82]. On the macro scale this leads to an increase of the turnover rate, as the time of reactivation of the enzyme is shorter. [83]

2.6 Quality Determining Parameters of Horsradish Pungency

Most of the people in the cultivation areas buy fresh roots, as freshly grated horseradish is inaccessible in its pungency for processed horseradish products. However, often people are disappointed as some roots only show a slight or no pungency at all. Indeed, the quality of horseradish from the point of view of the consumer is mainly related to the pungency of the product. The strong variation in the pungency of horseradish is therefore a problem for the industry, as they need to have a constant product quality. But so far only a limited number of parameters influencing the pungency of horseradish products has been studied. *Armoracia rusticana* belongs to the family of the *Brassicaceae*, to which brussels sprouts as well as cabbage, broccoli, cauliflower, rape seed and many others belong. For these other members of the family of the *Brassicaceae* plenty of literature can be found, dealing with parameters influencing their amount of glucosinolates. This scientific interest was fanned, on the one hand, by studies which found a toxic, goitrogenic and antinutritional effect of glucosinolates for animals [84] and on the other hand by studies which showed an anticarcinogenic effect of glucosninolates and their break down products [85]. Therefore plenty of studies tried to examine the parameters, which can be altered in order to influence the amount of glucosninolates in these plants. Some of them with the aim to reduce the amount of glucosinolates, e.g. in rape seed meal to increase the acceptance by animals as a nutritional source [86] and others to keep the amount of glucosinolates as high as possible, in order to sell products with the additional health benefit of being anticarcinogenic. The small scientific interest for horseradish roots may be due to the small amounts consumed, due to its usage as a spice. Following, the parameters affecting the amount of glucosinolates in *Brassicaceae* plants are presented. A transfer of the results of members of the same family to horseradish seems reasonable according to the similar biochemical pathways.

2.6.1 Date of Harvest and Stage of Maturity

Studies of the growing cycle of turnips and cabbage showed a correlation of the glucosinolate content with the date of harvest [87] and the stage of maturity [88]. In order to study the influence of the date of harvest on the glucosinolate content, turnips were sown in the beginning of May. After the turnips were fully grown in the end of July, the mature crops were harvested in distances of one month and the glucosinolate content was determined. As figure 2.14 shows, the glucosinolate content in the leaves increased continuously until February where a drop of the content was found. In the tuber the glucosinolate content was the highest in July and from there the content dropped to a plateau of approximately 50 % of the initial content. In February a further decrease to a second plateau with additionally 5 % less glucosinolates was recorded. These results are also representative for the amount of glucosinolates and their behavior in different plant components. According to the study the ratio between the different classes of glucosinolates is not affected by the date of harvest.

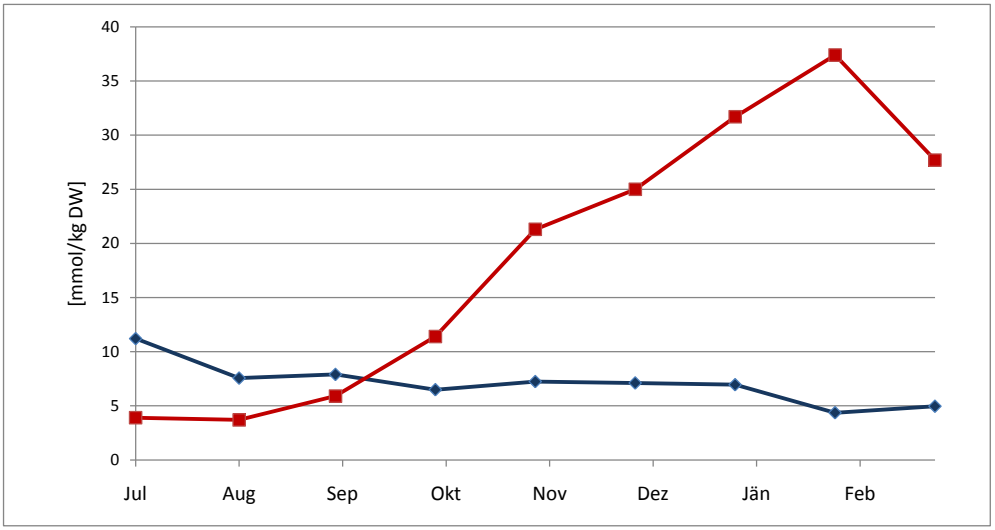


Figure 2.12: Depicted is the influence of the date of harvest on the total glucosinolate content in turnip leaves (red) and tubers (blue). The samples were collected after the turnips had fully grown, in distances of one month over eight months. [87]

The influence of the stage of maturity was also studied on cabbage. For this matter, cabbage was sown and after 70 days, when the first heads had formed, the crops were harvested in distances of two weeks and then analyzed. It became evident that with increasing stage of maturity, the amount of aliphatic glucosinolates increases only slightly, whereas the the content of indol glucosinolates drops significantly (see figure 2.13). As a conclusion of the results it can be said that there is an influence, but the extent of the influence of these two parameters depends on the class of glucosinolates and the plant component.

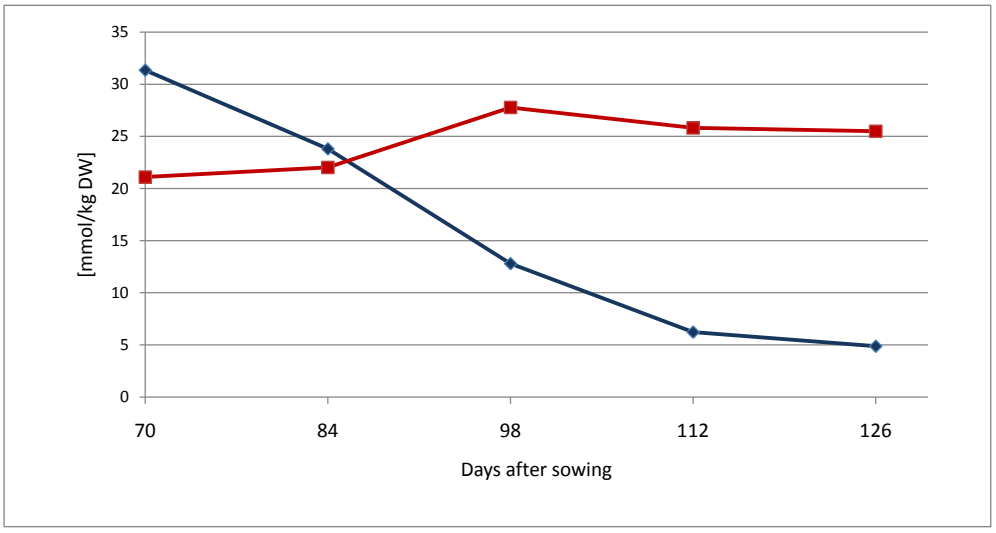


Figure 2.13: Depicted ares the influence of the stage of maturity on the aliphatic and indol glucosinolate content of cabbage. The samples were collected 70 days after sowing, when the first cabbage heads had started to form. A total of five samples was harvested, one every second week. [88]

2.6.2 Planting distance

In order to examine the influence of the planting distance on the glucosinolate content, a study by MacLeod *et al.* [89] planted brussels sprouts in three different distances of 30, 45 and 60 cm. The analysis of the ripened sprouts, showed that the sum of the volatile constituents increased with an increase of the planting distance. Planting distances of 60 cm led to a factor of 10 increase of the total amount of the volatile fraction compared to planting distances of 30 cm. This phenomenon was explained by the higher nutrition availability in soil, resulting from the lower competition for nutrition at bigger planting distances. However, a closer look at the different volatile substance classes reveals that the increase of the amount of the total volatile fraction with bigger planting distances is only caused by the substance class of the ketones. All other substance classes experience a decrease in their amount with increasing planting distances. In the case of isothiocyanates, planting distances of 60 cm had about 86 % lower amounts than distances of 30 cm. It is assumed that for the substance classes which show an increase of their amounts with decreasing planting distances, more low molecular precursors are synthesized for their biosynthesis caused by the stress in smaller plant distances.

2.6.3 Mechanical and Feeding Damages

By the simulation of mechanical wounding with needle stitches and feeding damages with flee beetles, a study by Bodnaryk *et al.* [90] examined the influence of these parameters on the glucosinolate content of one-week-old seedlings of mustard and oilseed rapes. In presence of these damages an increase of the total glucosinolate content of up to 300 % could be observed. However, if the contribution of the different glucosinolate classes on the total amount is taken into account, it shows that only the class of indole glucosinolates is increased, whereas the other classes of aliphatic and aromatic glucosinolates are not affected. The effect is stronger the more often the plant is exposed to these biotic factors. The results of this study were also confirmed for other members of the *Brassicaceae* family. The mechanism behind these effects is still topic of research. Interestingly, although the plants show a significant increase of their robustness in presence of these stress factors, this cannot be ascribed to the stronger expression of indole glucosinolates. Studies showed that pests fed with indol glucosinolates had a bigger appetite [91], whereas aliphatic glucosinolates lead to a decrease of feeding damages [92].

2.6.4 Fertilizer

Hydroponic cultivation is a method which allows to study the effect of altering nutritional supplies on plant parameters. A study by Josefsson *et al.* [28] used this method in order to examine the effect of nitrogen and sulfate supply on the glucosinolate content of rape seed meal. He found that high amounts of sulfate led to an increase of the glucosinolate content, whereas high amounts of nitrogen led to a decrease of the amount of glucosinolates. 97 % of the glucosinolates in rape are members of the aliphatic class, which are mainly biosyntesized from the amino acid methionine as e.g. sinigrin. On the other hand methionine itself needs sulfate for its biosynthesis. Therefore low contents of glucosinolates in association with low sulfate fertilization can be explained by a limitation of the biosynthesis of methionine by sulfate and due to that by a limitation of the synthesis of glucosinolates from methionine. Further, also the biosynthesis of cystein is linked to the availability of sulfate and cystein itself is needed for the biosyntesis of the sulfate linkage between the glucose and aldoxim moiety in the core structure (cf. chapter 2.2). In the case of nitrogen, the decrease of the glucosinolate content is also triggered by an intervention into biochemical pathways. The effect of nitrogen can simply be denoted as dilution. In other words, at high nitrogen supply, other plant constituents are produced in higher amounts than glucosinolates. On a biochemical level nitrogen leads to a more extensive use of the products of the citric acid cycle for the production of proteins rather than for carbohydrates. Therefore the biosynthesis of glucosinolates is limited by the availability of glucose at higher nitrogen levels.

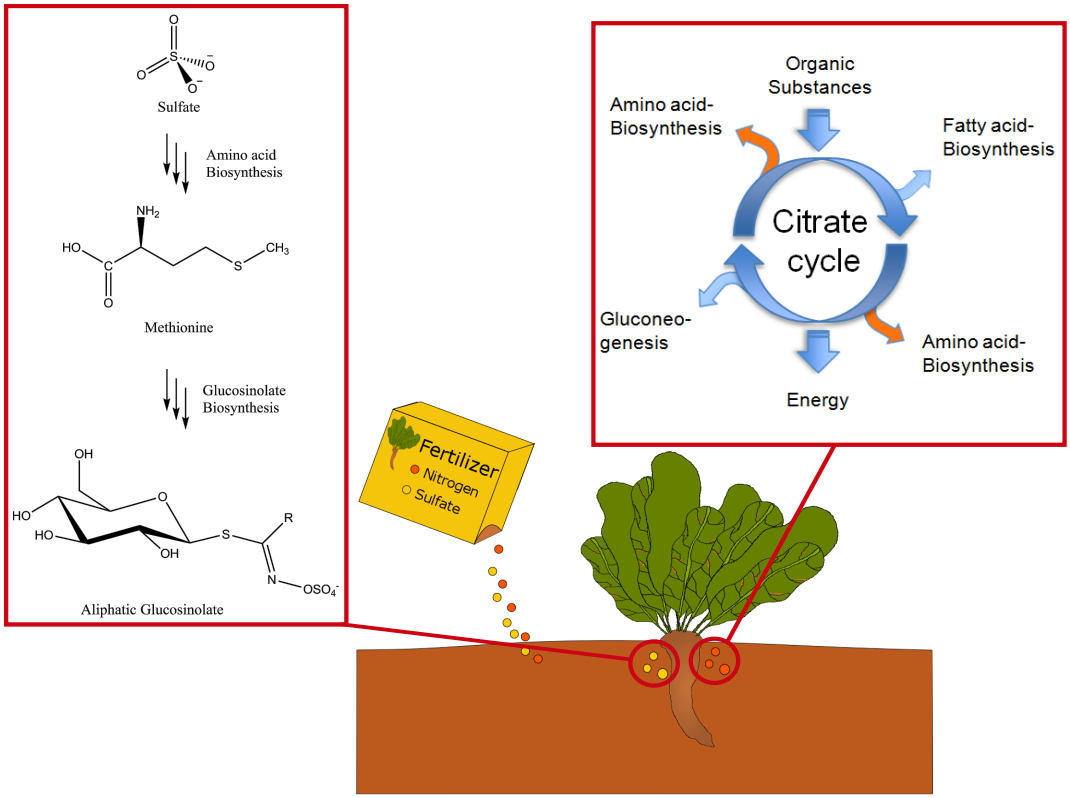


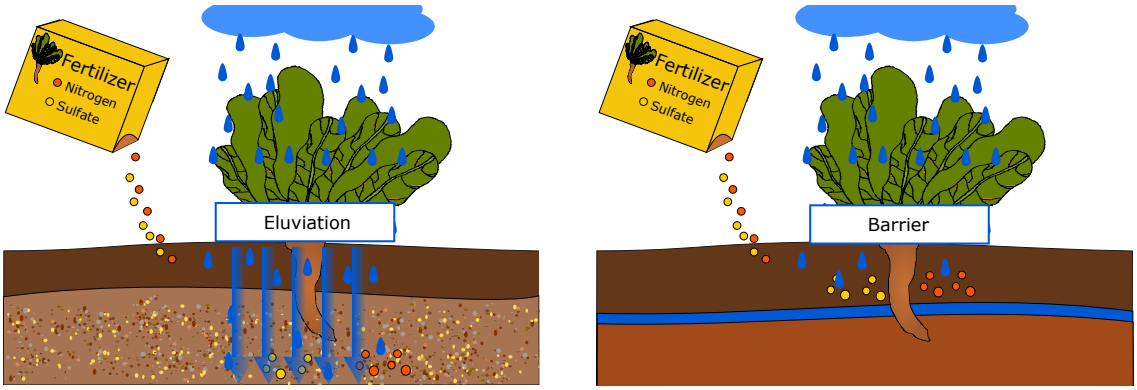
Figure 2.14: The level of nitrogen and sulfate in soil effects the glucosinolate expression in rape. The increase of the glucosinolate content at high sulfate levels can be explained by the availability of sulfate for the biosynthesis of methionine, which itself is a precursor to the biosynthesis of aliphatic glucosinolates. On the other hand, nitrogen leads to a more extensive use of the products of the citric acid cycle for the production of proteins than for carbohydrates, whereupon glucose is limiting the biosynthesis of glucosinolates.

2.6.5 Soil Conditions and Climate

A comparison [28] of rape grown on sandy and heavy soils showed, that fertilization with sulfate has a strong effect on sandy soils, whereas on heavy soils the glucosinolate content was only slightly affected. In the case of sandy soils, rain leads to the eluviation of sulfate and therefore to a reduction of the amount of glucosinolates (see figure 2.15a). On heavy soils clay acts as a natural barrier and prevents a strong leaching of sulfate (see figure 2.15b). As horseradish is typically cultivated on sandy soils, in order to allow the roots to easily penetrate into the soil, which leads to roots of high quality, a sulfate rich and nitrogen poor fertilization would increase the glucosinolate content.

A study of Ciska *et al.* [93] compared the glucosinolate content of different crops of the *Brassicaceae* family which were grown in two different years. The two years had great differences in their average temperature and their annual precipitation. The results showed that dry conditions combined with high temperatures correlate with increased glucosinolate contents.

On the contrary, wet conditions with lower temperatures lead to a decrease of the amount of glucosinolates. A similar study by Freeman *et al.* [94], which analyzed the influence of the water supply on the glucosinolate content, came to the conclusion that the lack of water leads to an increase of the amount of glucosinolates. Freeman and his group attributed this effect to an enhanced synthesis of amino acids and sugars, which act as precursors for the biosynthesis of glucosinolates. In comparison, Ciska *et al.* explained the effect to occur due to the eluviation of sulfate in rainy years. However, this explanation is not compatible with earlier studies which showed that sulfate mainly affects the class of aliphatic glucosinolates (cf. chapter 2.6.4). The study of Ciska *et al.* also showed a significant increase of the class of indol glucosinolates in the hot year. Based on the observation that indol glucosinolates are expressed intensively at mechanical or feeding damages (cf. chapter 2.6.3), it is possible that the dryness addresses the same biochemical signaling chain as the stress factor of mechanical and feeding damage.



(a) sandy soils **(b)** heavy soils

Figure 2.15: Represented is the effect of the soil conditions on the glucosinolate content. In the case of sandy soils, eluviation of sulfate leads to a decrease of the amount of glucosinolates, whereas in the case of heavy soils, clay acts as a natural barrier against the leaching of sulfate and therefore fertilization is of less importance on heavy soils.

2.6.6 Genotype

In figure 2.6 the results of Li *et al.* [8] are represented. He studied the influence of the genotype on the glucosinolate content in horseradish roots. From the results we can deduce that there is a significant influence of the genotype on the amount of glucosinolates found in horseradish. Depending on the genotype, the total amount of glucosinolates differs from 6 to 296 µmol/g dry weight. Further, the different classes of glucosinolates show a different dependence on the genotype, where aliphatic (sinigrin) and aromatic (gluconasturtiin) glucosinolates vary strongly

with the genotyp and indol (glucobrassicin) glucosinolates show only a slight dependence. Besides this, also the ratio between the different glucosinolate classes is affected by the genotype, as the highest amounts of aliphatic glucosinolates do not necessarily correlate with the highest amounts of aromatic glucosinolates. According to these results, the choise of the genotype is the most effective way among the presented influencing parameters to alter the pungency of horseradish products.

Table 2.5: This table shows a representative selection of the results of Li *et al.* [8], who studied the influence of the genotype on the glucosinolate content in horseradish roots.

Genotype	total GLS [µmol/g DW]	sinigrin [µmol/g DW]	glucobrassicin [µmol/g DW]	gluconasturtiin [µmol/g DW]	residual GLS
810-A	296	258	2.8	20.1	15
753-A	233	173	0.1	48.6	11.5
813-A	162	134	0.1	14.7	13.3
1590	152	112	1.1	28.9	10.5
493-A	134	124	0.6	2.8	6.7
1724	107	102	0.5	1.2	3.3
104-A	78	70	0.2	2.7	5.5
532-A	55	51	0.1	0	3.3
51-A	35	28	0.1	5.7	2.1
1717	21	16	0.3	3.8	1.4
811-A	10	8	0.1	0.1	1.3
53-A	6	5	0	0	0.7

GLS stands for glucosinolates
DW stands for dry weight

2.6.7 Phytohormones

Jasmonates are a group of phytohormones which are derivatives of jasmonic acid. Bodnaryk *et al.* [95] proved that the supply of jasmonates to rape or mustard crops shows the same effect as mechanical or feeding damage (cf. chapter 2.6.3). Apart form the higher effectivity of jasmonates, they also show an increase of the indol glucosinolate content, whereas aliphatic and aromatic glucosinolates were not affected. If a *Brassicaceae* plant is injured, jasmonates are released by a signaling cascade. The jasmonates then regulate the expression of proteinase inhibitor genes. It is assumed that the regulation of the synthesis of indol glucosinolates is regulated the same way. Contrary to the protease inhibitors, indol glucosinolates show no protection against pests. Up to now their function is still unknown.

2.6.8 Storage Temperature

Kosson *et al.* [9] studied the influence of the storage temperature and time on the isothiocyanate content of horseradish pastes. For the experiment, horseradish pastes were stored at three different temperatures for eight months and in distances of two months the isothiocyanate content was determined. In the first four months the results showed the biggest decrease of allyl and phenethyl isothiocyanate content, which then flattened drastically over the last months (cf. figure 2.16). Additionally, the results indicate a strong influence of the storage temperature, as at storage temperatures of 2 and 8 °C a significantly lower loss of the analytes occured compared to 18 °C. The authors explained the smaller loss of analytes at lower temperatures by a decrease of the enzyme activity of *myrosinase* with the temperature and therefore with a decelerated degradation of the corresponding glucosinolates. Additionally lower temperatures result in a decelerated self decomposition of the cells, which maintains the spatial separation of glucosinolates and *myrosinase*. In comparison, storage at - 85 °C leads to a significant loss of glucosinolates [96]. This is due to freeze-fracturing at this temperature, which leads to the elimination of the spatial separation and at thawing to the degradation of the glucosinolates by *myrosinase*.

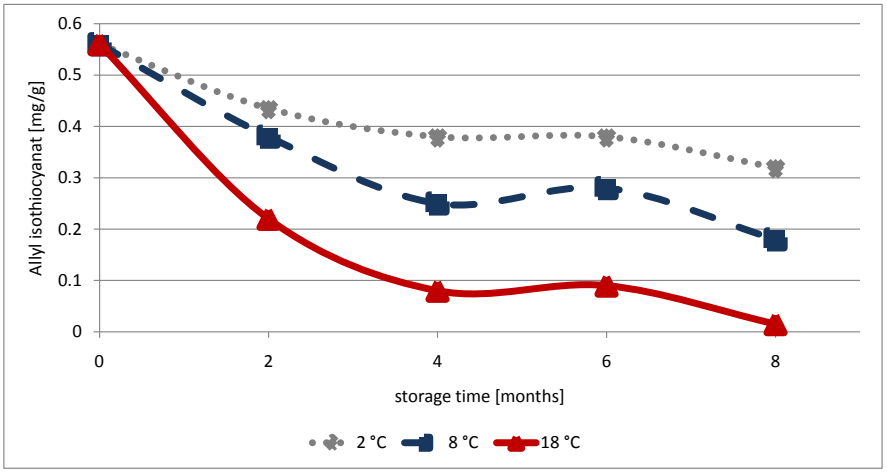


Figure 2.16: The diagram shows the results of the study of Kosson *et al.* [9], who examined the influence of the storage time and temperature (2, 8, 18 °C) on the glucosinolate content of horseradish pastes.

2.6.9 Crushing and Enzyme Activity

Crushing of *Brassicaceae* plants leads to the elimination of the spatial separation of glucosinolates and their degradation enzyme. As one would assume, the degree of crushing has an influence on the loss of glucosinolates, which was studied by Song *et al.* [96]. When broccoli, brussels sprouts, cauliflower or cabbage are cut into 5 mm cubes or 5x5 mm squares, within the subsequent 6 hours, 75 % of the total glucosinolate content is lost. In comparison, slicing of 4 cm cubes or

4x4 cm squares was only accompanied by the loss of 10 % of the overall glucosinolate content. If now horseradish roots are crushed into pastes, after the first 15 minutes the glucosinolate content declines below 2 % of the initial amount and within the next 15 minutes reaches zero [8] (see figure 2.17).

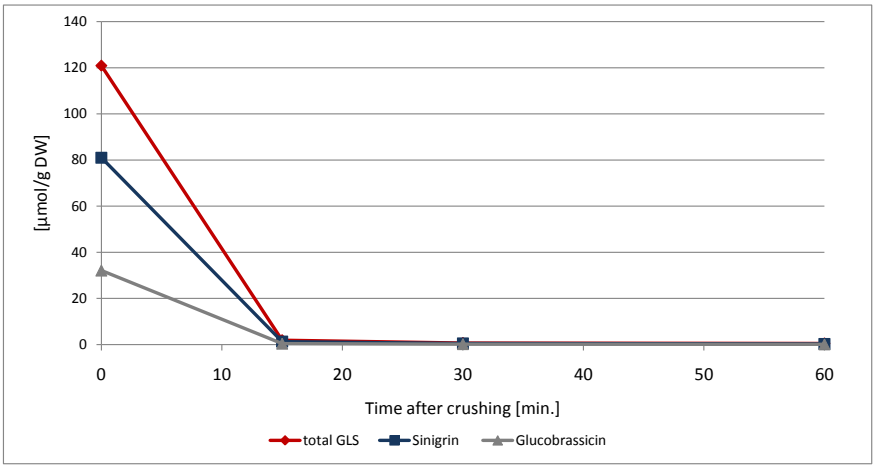


Figure 2.17: Shown are the results of the study of Xian *et al.* [8], who examined the decrease of the glucosinolate content after horseradish roots were crushed into pastes.

Similar to the glucosinolate content, also the enzyme activity of *myrosinase* decreases rapidly. Within the first 15 minutes the *myrosinase* activity declines by 67 % (see figure 2.18). This effect was explained by the inactivation of the enzyme by the hydrolysis products, where, according to Shikita *et al.* [97], only sulfate shows a significant inhibition of *myrosinase*. Additionally, the enzyme activity also depends on the genotype and, interestingly, high enzyme activities are not necessarily found in genotypes with high glucosinolate contents (cf. table 2.6).

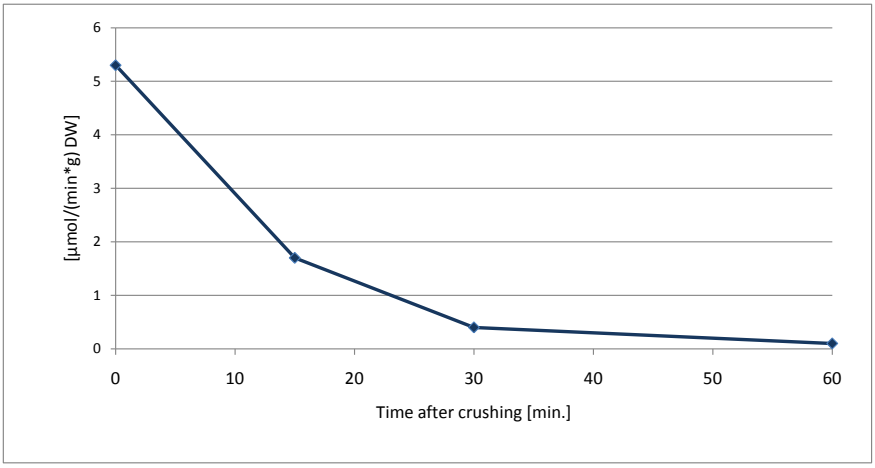


Figure 2.18: Represented are the results of another study of Xian *et al.* [8], in which he examined the course of the enzyme activity of *myrosianse* after horseradish roots were crushed into pastes.

Table 2.6: This table shows the results of Xian *et al.* [8], who studied the influence of the genotype on the *myrosinase* actvitiy in horseradish roots.

Genotyp	total GLS [µmol/g DW]	*myrosinase* activity [units/ g DW]	Genotyp	total GLS [µmol/g DW]	*myrosinase* activity [units/ g DW]
810-A	296c	4.3	104-A	78	25.7
753-A	233	21.1	125-A	75	57.1
113-A	191	10	1573	56	3.2
239-A	175	22.5	532-A	55	2.8
813-A	162	3.3	856-A	40	44.5
Bohemian	154	1.2	51-A	35	23.1
1590	152	10.7	1069	33	9.5
106-A	152	33.3	789-A	27	22.7
196-A	140	8.7	777-A	20	1.7
493-A	134	50.4	244-A	11	21.5
819-A	131	23	811-A	10	8.2
502-A	127	13.2	53-A	6	21.6
1724	107	26.3	Big-Top-W.	2	20.7

GLS stands for glucosinolates
DW stands for dry weight

2.7 Analytical Methods

The following chapter provides a summary of a literature research for methods applied so far for the determination of glucosinolates, isothiocyanates and organic acids, with a focus on citric acid. There is no claim for completeness.

2.7.1 Methods for the Determination of Glucosinolates

As glucosinolates are built up by three different parts, a β-D-thioglucose group, an anionic sulphonated oxime group and a characteristic side chain, there are three targets for the analysis of glucosinolates. Hydrolysis breaks glucosinolates into 3 compounds and by determination of the released glucose or sulphate content, the total amount of glucosinolates is accessible. In order to determine the content of different glucosinolates, either the corresponding side chain degradation products (e.g. isothiocyanates) have to be used as analytical targets or the glucosinolate structure has to be maintained. To maintain the structure for analytical samples, preparation conditions have to be chosen very carefully, as injuring of the cells results in the release of myrosinase and therefore in hydrolysis. Further, thermal degradation occurs at temperatures above 125 °C. According to this, plant samples have to be stored in an uninjured state at low temperatures before analysis [98]. Subsequently the known methods for glucosinolates determination are presented.

Determination of Glucosinolates via their Hydrolysis Products Glucose or Sulfate

For the determination of glucosinolates via their hydrolysis products glucose or sulfate, only the total amount of glucosinolates is accessible, because of the loss of the side chain information during hydrolysis. A further disadvantage is given when the matrix contains endogenous glucose or sulfate, as this leads to an overestimation of the glucosinolate content. For rapid testing, methods have been developed based on this knowledge and have their eligibility for this use (see table 2.7).

Table 2.7: Summary of methods for glucosinolate determiantion by measuring glucose (G) or sulfate (S) received from *myrosinase* hydrolysis.

Method	Analyte	Description	Literature
Thymol test	G	Strong acid causes dehydration and ring closure of glucose to furaldehyde. By reaction with thymol a red colored product is formed.	[99]
Colorimetry	G	Colorimetric measurement of glucose after specific oxidation including glucose oxidase, peroxidase and a chromogen.	[100]
Test paper	G	Liberated glucose can be measured using a test paper and determination of the color intensity with a pocket reflectometer.	[101]
Sulfate rapid test	S	The sulfate rapid test is based on measuring the turbidity of a barium sulfate precipitate.	[102]
Ion chromatography	S	Sulfate ions are separated from other ions found in the extract by ion chromatography and are detected by a conductivity detector	[103]
Optical biosensing	G	Glucosinolates are hydrolysed on a myrosinase-immobilized eggshell membrane. The formed glucose is measured by an optical glucose biosensor consisting of a glucose oxidase immobilized eggshell membrane and an oxygen sensitive optode membrane.	[104]
X-ray emission spectroscopy	S	Sulfate is precipitated with barium chloride and the formed barium sulfate crystals are measured with X-ray emission spectroscopy.	[105]

Determination of Glucosinolates with Enzyme-Linked Immunosorbent-Assays

The first ELISA (Enzyme-Linked Immunosorbent-Assay) test adopted for sinigrin and progoitrin was reported by van Doorn *et al.* [106]. Samples were prepared by extraction of glucosinolates from plant samples with 2 % phosphoric acid followed by neutralization and an additional heat treatment in order to remove inactivated proteins. The ELISA test was developed as a double-bond test (sandwich configuration), which means that the antigen, in this case sinigrin

and progoitrin, is first bound to the specific polyclonal antibody linked to the immuno-assay and then a second specific antibody binds from the other side. For determination, the second antibody is labeled, for instance, with a fluorophore or radioactive. Antibodies were received from treating rabbits with synthesized conjugates of sinigrin- and progoitrin-BSA or ovalbumin. Compared to the time consuming sample preparation for HPLC detection of glucosinolates, ELISA is a fast and reliable quantification tool, where the number of samples plays a subordinate role. By synthesis of other glucosinolates conjugates, it should be possible to generate further antibodies to extend the amount of glucosinolates accessible for quantification by ELISA.

Determination of Glucosinolates with Spectrophotometric Methods

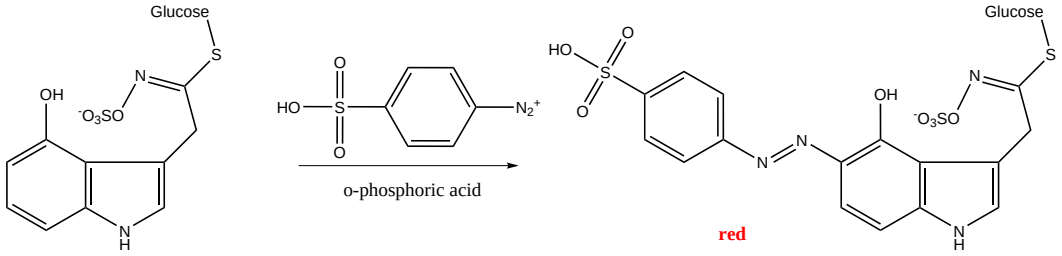


Figure 2.19: Reaction of 4-hydroxyglucobrassicin with diazotized sulfanilic acid to a red colored chromophore, which allows the determination by spectrophotometric methods.

Spectrophotometry requires a light absorbing compound, but glucosinolates except, the one with aromatic moieties (UV absorption), lack this property. A specialized method uses diazotized sulfanilic acid in presence of o-phosphoric acid to form a red colored coupling compound with indole glucosinolates, with a maximum of absorption at 510 nm. This method was developed as a cheap alternative for HPLC, for instance for the use of mass selections in breeding programs [107].

Determination of Glucosinolates with Near-Infrared Spectroscopy

Glucosinolates have the capacity to absorb infrared radiation. NIRS (Near-Infrared Spectroscopy) uses this ability and is capable of measuring a wide range of different glucosinolates, based on to the characteristic IR vibration bands of the side chains. Also, NIRS is able to cover a broad concentration range, where in comparison glucose tests are only able to determine the glucosinolate content of plant samples, which contain a certain amount of glucosinolates. A further advantage is the reduced sample preparation, which is minimized to placing dried samples into the NIRS measurement cell [108].

Determination of Glucosinolates with Gas Chromatography

According to the ionic nature of glucosinolates, they are not directly accessible by GC. Pre-column derivatisation including desulfonation and silylation has been established as a common method to form volatile glucosinolate derivatives [109–116]. Silylation further has the advantage of shorter analysis times and lower running costs in routine applications. As separation system, partition chromatography has been generally accepted. Following, the derivatisation method reported by Slominski *et al.* [116] is described exemplarily.

- Inactivation of myrosinase and extraction of glucosinolates:
  Defatted seeds or commercial meal were heated on a boiling water bath for 10 minutes, then mixed with hot water (95 °C) and held over the water bath for another 3 minutes Subsequent, the samples were immediately cooled and allyl or benzyl glucosinolate added as internal standard.

- Purification of the extract:
  Organic acids can be removed from the extracts by precipitation with barium and lead acetate when added prior to centrifugation (glucosinolate lead salts only precipitate in methanol). The obtained supernatant was applied to ion exchange for isolation of glucosinolates. The supernatant was loaded onto a DEAE sephadex A-25 column and washed with pyridine acetate. Then, a sulfatase solution was added onto the column and left overnight at room temperature. In order to elute the desulfo-glucosinolates, the column was rinsed with water.

- Derivatisation:
  Prior to derivatisation, water was removed and the residual redissolved in pyridine. After the addition of MSTFA (N-methyl-N-trimethylsilane-trifluoroacetamide) and TMCS (trimethylchlorosilane) to the purified extracts, the vials were capped, homogenized and heated for 20 minutes at 120 °C.

- Gas Chromatography:
  Silylated desulfo-glucosinolates were separated using gas liquid chromatography with helium as carrier gas and were determined with a flame ionization detector (FID). Separation included a temperature program starting at 200 °C for 4 minutes followed by a gradient of 5 °C/min. to 275 °C.

A drawback of this method is the partial loss of indole glucosinolates, due to heat treatment for myrosinase deactivation. The loss during dry heat treatment was independent of time and averaged around 15 %, whereas wet heat treatment decreased the indol glucosinolates content by time, after 10 minutes to about 75 %. Aliphatic glucosinolates were not affected at all. Another disadvantage is that large volumes of water are needed to guarantee full recovery of derivatised glucosinolates from the ion exchange column. Thies [112] could show that water for

extraction of glucosinolates from defatted meal had the highest efficiency compared to methanol, ethanol or mixtures of them with water. The maximum amount of glucosinolates was extracted after 10 minutes with boiling water (95 °C). Longer treatments neither enhanced nor decreased the yields of glucosinolates in the extracts. Silylated compounds can cause deteriorations of the FID-electrodes when serial analyses are made. Condensation of SiO_2 may lead to electric short circuits in extreme cases. In order to solve this problem, methylsilylhexafluorobutyramide (MSHFBA) can be used as potent surrogate for MSTFA. Pyridine, which is used for silylation, has toxic properties, but replacement by other non- or less-toxic solvents is not possible as pyridine shows a catalytic effect on silylation.

Determination of Glucosinolates with High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has established itself as the most appropriate method for glucosinolate determination, mainly because there is no need to convert glucosinolates into volatile derivatives and determination of thermally unstable glucosinolates as indol species is less critical. The three methods of desulfation followed by reversed-phase (RP) HPLC, ion-pair (IP) HPLC and hydrophilic interaction chromatography (HILIC) have proven to be the most appropriate HPLC techniques for glucosinolate determination and are described subsequently.

Reversed-Phase High Performance Liquid Chromatography

The most widely used technique for glucosinolate determination is reversed-phase HPLC of desulfated glucosinolates. Exemplarily the method of Kushad *et al.* [117] is described. The most distinctive difference to other methods [7, 118–120] is the use of water for glucosinolate extraction, in comparison the others who used a mixtures of 70 % methanol as extraction solvent.

- Pre-sample preparation:
  Plant samples were frozen with liquid nitrogen and lyophilized before grinding to a fine powder. The freeze-dried powder was stored at – 20 °C.
- Extraction:
  For inactivation of *myrosinase* and the glucosinolate extraction, the freeze-dried samples were first dry heated in glass tubes on a heating block at 95 °C for 15 minutes. Then boiling deionized water was added as well as an internal glucosinolate standard and the sample was heated for another 5 minutes. Following, the extracts were cooled on ice and centrifuged. The supernatants were then used for desulfation.
- Desulfation:
  A barium acetate solution was added to the extract a and the mixture layered on a DEAE Sephadex (A-25) column. Desulfation was induced by the addition of arylsulfatase and

was proceeded for 18 hours. The desulfated glucosinolates were recovered by rinsing the column with water.

- HPLC separation coupled to UV detection:
  Desulfated glucosinolates were separated on an RP-18 column at 32 °C by applying a linear gradient of 0 - 20 % acetonitrile in water. The flow rate was adjusted at 0.8 ml/min.

Ion-Pair HPLC and HILIC of intact glucosinolates

Reversed-phase HPLC suffers from incomplete desulfation, due to the influence of the pH on the enzymatic activity, problems of defining the reaction period and determination of the enzyme concentration during desulfation [98]. Without desulfation, the ionic character of glucosinolates has to be handled and this is possible by using IP-HPLC [121–125] or HILIC [126–128]. Generally the use of IP-HPLC allows the separation of solute ions with reversed-phase chromatography in presence of lipophilic ions in the mobile phase. Coupling to these leads to the formation of ion pairs according to the opposite charge. The high charge of the sulfate group is therefore neutralized by the ion pairing agent [127]. Different solvent systems have been tested:

- Ammonium acetate, phosphate and sulfate with water [121]
- Tetrapentyl ammonium bromide in phosphate buffer with water and an acetonitrile gradient [122]
- Triethylamine and formate buffer with water and a methanol gradient [122]
- Trifluoroacetic acid with water and a methanol gradient [122–125]

The system with the best performance turned out to be a gradient with water and methanol both containing 0.1 % v/v trifluoroacetic acid. Following, the extraction and chromatography conditions used by Mellon *et al.* [122] are described exemplarily.

- Extraction of intact glucosinolates:
  With the goal to inactivate myrosinase, seeds were dried at 100 °C for 24 hours in an oven. The seeds were then rapidly homogenized in presence of 70 % v/v methanol and sinigrin as internal standard was added. Again the homogenate was heated to 70 °C for 30 minutes and vortexed in an interval of 5 minutes. Following, the homogenate was centrifuged and filtered prior to the replacement of methanol by water, which turned out to be essential to ensure entire peak separation.
- IP-HPLC:
  A C18 reversed-phase column was connected in series to a pre-column. As mobile phase, water and methanol with 0.1 % v/v trifluoroacetic acid were used for gradient elution, starting with 100 % water and ending up with 100 % methanol.

Another possibility to separate glucosinolate ions is HILIC. Like normal phase chromatography, HILIC uses traditional polar stationary phases such as silica, though the mobile phase is similar to one used by reversed phase chromatography. This allows HILIC to separate charged substances, with the advantage that the use of water containing eluents guaranties the solubility of highly polar substances compared to normal phase chromatography [129]. Following, representative for HILIC, the method published by Troyer *et al.* [127] is described.

- Extraction of intact glucosinolates:
  Grinded and homogenized seeds were extracted using a triple solvent consisting of equal volumes of acetonitrile, dimethylformamide and dimethylsulfoxide at – 50 °C on a dry-ice-ethanol bath. After centrifugation, the supernatants were dried and the residuals resuspended in the mobile phase.
- HILIC:
  Separation of intact glucosinolates was achieved by using a polyhydroxyethyl aspartamide column and an isocratic mobile phase with approximately 85 % v/v acetonitrile in water and 30 mM ammonium formate.

Nevertheless, if just one system is used, IP-HPCL or HILIC, it is not possible to detect the whole range of known glucosinolates. IP-HPLC has its strength in separating non-polar glucosinolates and HILIC is an appropriate tool for the separation of polar glucosinolates. But because they are complementary to each other, the concerted use allows the analysis of the whole range of glucosinolates. A new approach reported by West *et al.* [130] allows the simultaneous determination of both polar and non-polar glucosinolates. For this purpose, a C18-bonded silica column, where the residual silanol groups were hydrophilically endcapped, was used in combination with a mobile phase gradient of 50 mM ammonium acetate to a mixture with ethanol (80:20).

2.7.2 Methods for the Determination of Isothiocyanates

Isothiocyanates are volatile and suffer from instability, therefore methods for sample preparation and determination have to be cleverly worked out. The methods developed up to now are very time consuming and laboratory intensive due to complex sample preparation, including hydrolysis of glucosinolates to the corresponding isothiocyanates, extraction, isolation and concentration steps. This chapter includes a precise description of the crucial parameters of sample preparation and deals with the different techniques for the determination of isothiocyanates.

Hydrolysis of Glucosinolates to Corresponding Isothiocynates

As the biologically active isothiocyanates are stored in form of highly stable glucosinolates, this precursor compounds have to be fully hydrolyzed in order to allow the determination of

isothiocyanates. Three methods are known [83, 98], the hydrolysis by the enzyme *myrosinase*, acid hydrolysis and degradation of silver-labeled glucosinolates in presence of iodide ions at an pH of 7. For these methods, the right reaction conditions have to be found in order to prevent the formation of other degradation products than isothiocyanates (cf. chapter 2.5.3).

Acidic Hydrolysis

Sivakumar *et al.* [131] used acid hydrolysis to convert glucoraphanin into sulforaphane. For this purpose lyophilized plant samples were treated with 0.1 M hydrochloric acid. The suspensions were incubated for 2 hours at 42 °C in a shaking water bath. Further sample preparation started with the extraction of the released sulforaphane with dichloromethane. Acid hydrolysis prior to extraction had the advantage of an increase in the yield of sulforaphane. Additionally the method stands out due to its faster hydrolysis rate compared to other hydrolysis procedures and the higher recovery of sulforaphane.

Degradation of Silver-Labeled Glucosinolates in Presence of Iodide Ions at pH 7

Treatment of glucosinolates with $AgNO_3$ (aq.) leads to the replacement of the glucose moiety by silver and to the formation of the silver salts of aglucones. In presence of iodide ions, the silver salts of aglucones undergo decomposition to the corresponding isothiocyanates (pH 7) or to the cyanide derivative (pH 4) depending on the pH [83]. Also sodium thiosulfate can be used instead of iodide. Then the decomposition results mainly in the formation of isothiocyanates with some nitrile side products.

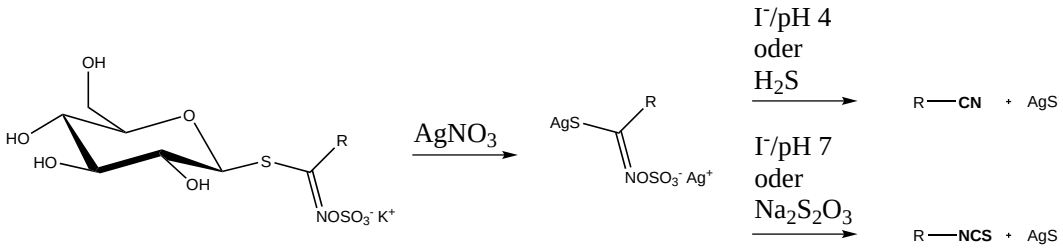


Figure 2.20: In presence of $AgNO_3$, glucosinolates form silber-labeled aglucons, which can be degraded to either the corresponding isothiocyanates or nitriles by the choice of the degradation conditions.

Endogenous and Exogenous Hydrolysis with *Myrosinase*

There are two possible ways of *myrosinase* hydrolysis, namely the endogenous *myrosinase* method or the exogenous *myrosinase* method, where the glucosinolates are isolated prior to enzymatic hydrolysis. In presence of *myrosinase*, glucosinolates are converted into aglucon

intermediates, which then, depending on the reaction conditions, degrades into the corresponding isothiocyanates, thiocyanates or nitriles. The reaction conditions leading to the different degradation products were discussed in chapter 2.5.3.

Spectrophotometry for Isothiocynate Determination

Spectrophotometric methods make use of the high reactivity of isothiocyanates. Different derivatisation methods have been developed to allow the quantitative formation of stable light absorbing reaction products. Following, two derivatisation methods are represented.

Derivatisation of Isothiocyanates with Thioglycolic Acid to Rhodanines

Prior to derivatisation, dried and grounded seeds of *Brassicaceae* plants were extracted with methanol, followed by filtration and reuptake in water. The so obtained glucosinolate extracts were hydrolyzed to the corresponding isothiocyanates by adding citrate buffer (pH 6.5), ascorbic acid and a solution of the enzyme *myrosinase*. Then, by addition of aceton, borate buffer (pH 10) and the derivatisation reagent thioglycolic acid, the isothiocyanates were converted into rhodanines, still representative for each isothiocyanates, because the R moiety stays in the rhodanine. After adding hydrochloric acid, extraction with ethyl acetate and replacement of the solvent by acetic acid, the obtained solution was used for direct qualitative or quantitative determination of the corresponding rhodanines by spectrophotometry (λmax 260 and 295 nm).

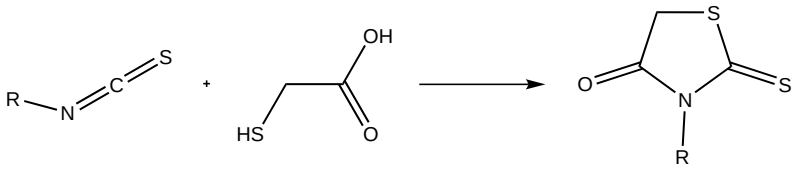


Figure 2.21: Derivatisation of isothiocyanates with thioglycolic acid to the corresponding rhodanines.

Derivatisation of Isothiocyanates with 1,2-Benzenedithiole to 1,3-Benzodithiole-2-thione

Glucosinolate isolation prior to the derivatisation started with the extraction of lyophilized plant samples with acetonitrile. Then acetonitrile was substituted by methanol and preparative RP-HPLC used for further purification. For hydrolysis of glucosinolates, sodium phosphate buffer (pH 6.1) was added to the obtained extracts, followed by the addition of a solution of *myrosinase*. After a defined time, aliquots were removed and mixed with ethanol and transferred into a screw-top vial which contained 1,2-benzenedithiole in methanol and potassium phosphate (pH 8.5) as buffer. Spectroscopic determination of the concentration of 1,3-benzodithiol-2-thione,

the reaction product of isothiocyanates with 1,2-benzenedithiol with an absorption maximum at 365 nm, is a competent tool to determine the total isothiocyanate content. An advantage of this technique is the quantitative and also quick reaction with almost all isothiocyanates, the stability of the formed cyclocondensation products and that thiocyanates and cyanates do not interfere with isothiocyanates during the reaction.

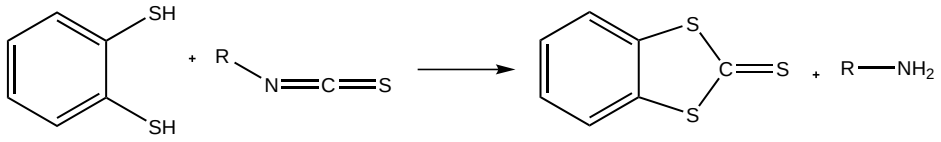


Figure 2.22: Derivatisation of isothiocyanates with 1,2-benzenedithiole to 1,3-benzodithiole-2-thione, which is accompanied by the loss of the R moiety.

Gas Chromatography for Isothiocyanate Determination

Gas chromatography is an appropriate tool for the determination of isothiocyanates, as they are volatile. For quantification and identification of isothiocyanates, FID and MS detectors are commonly used. Drawback is the thermal instability of isothiocyanates at high temperatures, which may cause troubles in analysis. For instance, comparison of split to split-less mode to on-column injection showed that 80 % of sulforaphane were decomposed to 3-butenyl isothiocyanate [132]. Various methods for sample preparation have been developed based on the analytical question and the starting product, e.g. mustard, plant samples or seeds. Following, a summary of sample preparation methods is given.

Headspace Technique for Isothiocyanate Extraction

A method based on headspace extraction followed by GC-MS of allyl isothiocyanate from horseradish containing products was published by Korb *at al.* [133]. Defined amounts of grounded horseradish and cocktail sauce containing horseradish samples were transferred into serum bottles and sealed with a rubber septum. Samples were equilibrated at 40 °C for 30 minutes, before headspace samples were removed and analyzed by GC-MS. The study further proved a high matrix effect by adding sugar to standard solutions of allyl isothiocyanate in water. Allyl isothiocyanate has, according to its hydrophobicity, a low solubility in water, which is further decreased when sugar is added. But recovery tests suggested that by comparing spiked and un-spiked samples, it should be possible to determine the quantity of allyl isothiocyanate in unknown matrices. HS-SPME is not only an appropriate technique do determine the allyl isothiocyanate content, also all other isothiocyanates in horseradish can be measured as the method of Tomson *at al.* [4] proved.

Peruga *at al.* [134] described a method for the determination of methyl isothiocyanate by HS-SPME with a GC-MS/MS system. Their initial goal was the determination of the highly toxic and volatile methyl isothiocyanate in soil and water, as it is the main degradation product of the soil disinfectant metam sodium. For the HS-SPME, water (5 mL, 20 % NaCl) or soil (1 g, 180 µL deionized water) were transferred into a glass vial and the fiber was exposed to the headspace for 20 minutes at 30 °C. Using a hot injector, thermal desorption was performed at 300 °C for 3 minutes in splitless mode. Six fibers were tested on their adsorption performance and carboxen-poly(dimethylsiloxane) (CAR-PDMS) was found to have the highest response to methyl isothiocyanate. Further they reported an increase of methyl isothiocyanate in soil at higher temperatures, whereas lower temperatures and higher NaCl contents as well as increased steering had a positive effect on the methyl isothiocyanate content of water samples. GC-MS/MS was used for detection because compared to SPME it offers the advantage of simple and solvent-free sample preparation, based on the high selectivity and sensitivity of tandem mass spectrometry.

Another system which uses HS-SPME was published by Jain *at al.* [135]. They determined aromatic primary amines after derivatisation to isothiocyanates via dithiocarbamates with HS-SPME followed by GC-MS analysis. River water, food color, ice cream powder or soft drink samples were mixed with the derivatisation reagents and heated to 60 °C for 10 minutes while stirring. Then, at the same temperature, the formed isothiocyanates were adsorbed with an SPME fiber and thermally desorbed for 5 minutes at 250 °C in the injector of the GC-MS. Again fibers where tested on their adsorption performance and DVB/CAR/PDMS (50/30 µm) reached the best adsorption values. Evaluation of the best adsorption temperature, however, showed an optimum at 60 °C, maybe because of the derivatisation reaction. Compared to Peruga *et al.* salt addition had no positive effect on the extracted amount of isothiocyanates, which was explained by the formed non-polar isothiocyanates. Further, an increase of desorption temperature as well as an elongation of desorption time did not lead to an increase of the peak areas.

Solvent Extraction for Isothiocyanate Extraction

A simple solvent extraction method for the determination of the isothiocyanate content in mustard was reported by Marton *et al.* [136]. Isothiocyanates of mustard were extracted by using n-hexane as solvent. The two phases were separated by centrifugation and the supernatants were filtered before the extracts were directly analyzed with GC-MS/MS.

A method for solvent extraction with prior endogenous *myrosinase* hydrolysis of glucosinolates was published by Chiang *et al.* [132]. For the endogenous *myrosinase* hydrolysis, fresh plant samples were homogenized for 5 minutes in 50 °C warm distilled water using an

Omni 5000 mixer. Then the homogenates were centrifuged and the supernatants filtered. The hydrophobic isothiocyanates were then extracted with dichloromethane, the organic phase dried over sodium sulfate, filtered and the extracts concentrated to a defined volume prior to GC-MS injection. The study could further show the thermal degradation of sulforaphane to 3-butenyl isothiocyanate. Comparison of split/splitless injection to on-column injection indicated that about 80 % of sulforaphane were degraded in the injector due to the high temperatures. But neither the temperature can be reduced, because this could result in deposition of material on the column, nor on-column and programmed temperature vaporizing injection are possible solutions. However, by adjusting a high constant flow on the injector and by choosing the right injector geometry e.g. small and direct inlet liner, the dwell time of the sample was shortened, whereupon the degradation of sulforaphane could be reduced to 5 %. A similar method including endogenous *myrosinase* hydrolysis and solvent extraction was reported by Li *et al.* [137]. The objective of this study was to optimize hydrolysis and extraction conditions as well as the conditions for GC analysis in order to improve the qualitative and quantitative validity of the obtained results. Subsequent, the results of the study are summarized.

Effects on the extraction yield:

- Storage temperature:
  Tests have shown that lower storage temperatures and therefore low temperatures at the release of *myrosinase* at grinding lead to a higher concentration of isothiocyanates in the extraction solutions. At 37 °C isothiocyanates are almost released instantaneously from glucosinolates by endogenous *myrosinase* hydrolysis. In comparison, at 4 °C the *myrosinase* activity is much lower, whereby less isothiocyanates are volatilized to air and less isothiocyanates are decomposed by non-enzymatic reactions [138] before extraction with dichloromethane.

- Time between grinding and filtration:
  Extraction of isothiocyanates with dichloromethane should be done as quickly as possible after grinding, in order to prevent the loss of isothiocyanates. However, it could be shown that delays in extraction of up to 10 minutes have no significant effect on the isothiocyanate concentration in the extract. But if 10 minutes were exceeded, the concentration decreased very fast.

- Time for endogenous *myrosinase* hydrolysis:
  Because *myrosinase* activity is slowed down at 4 °C, samples were sealed and held at 37 °C while shaking. In the first hour a drastic increase of isothiocyanate concentration could be observed, which then plateaued to a constant lower value after 2 hours. This phenomenon was explained by evaporation of dichloromethane and a delayed equilibration of isothiocyanates between the liquid and gasous phase.

- Storage time after hydrolysis at low temperature:
  After hydrolysis, *myrosinase* was separated by centrifugation. As mentioned above, at 37 °C a significant amount of dichloromethane evaporated and the vapor contained a defined amount of isothiocyanates according to the equilibrium. Cooling to 4 °C prior to centrifugation resulted in an increase of up to 20 % of the isothiocyanate concentration in the extract because the loss at the transfer step to the centrifugation is prevented by condensation of dichloromethane in the headspace. Constant values were achieved by cooling for 1 hour.
- Dehydration:
  For dehydration sodium sulfate was used. Increasing amounts of sodium resulted in a decrease of isothiocyanates. Perfect conditions were found to be low sodium sulfate amounts (0,25 g/5 mL) and 30 minutes stirring.

Gas chromatography settings:

- The perfect separation conditions were found to be 80 °C for the starting column temperature, which is held for 2 minutes followed by a constant increase of 5 °C/min. to 160 °C. A constant starting temperature was needed to separate allyl isothiocyanate peaks from the solvent peak. Further, constant temperature programs were not able to separate all peaks and if the temperature gradient was too high, the base line was not smooth.

High Performance Liquid Chromatography for Isothiocyanate Determination

HPLC is another technique used for isothiocyanate determination. Methods have been developed for either determination of the total isothiocyanate content or the specific determination of individual isothiocyanates. The methods include derivatisation reactions as well as solid phase extraction (SPE) for sample preparation. For detection, normally HPLC is coupled with a DAD- (diode array detector), ELSD- (evaporative light-scattering detector) or MS-detector. Following, the different methods are discussed.

Derivatisation of Isothiocyanates for determination with HPLC-DAD

1,3-benzodithiole-2-thione derivatisation of isothiocyanates with 1,2-benzenedithiole was previously used for the determination of the total isothiocyanate content with UV/VIS-spectroscopy. By comparison to HPLC connected to an integrated UV/VIS detector, lower LODs could be achieved. Lyophilized and disintegrated plant samples were derivatised according to the procedure published by Zhang *et al.* [139]. Prior to chromatography, 1,3-benzodithiole-2-thione was extracted with dichloromethane. HPLC used an RP-C18 column in combination with an isocratic elution program (mobile phase 80 % methanol) for separation [98, 131].

By using ammonia for derivatisation of isothiocyanates to the corresponding thiourea derivative, the information of the specific R moiety of isothiocyanates is not lost. Song *et al.* [124] and Agerbirk *et al.* [140] used the thiourea derivatisation method for the determination of the concentration of the specific isothiocyanate after separation with HPLC. For detection they used tandem MS spectroscopy with electrospray ionization. For derivatisation, fresh plant samples were homogenized in presence of 2 M ammonia in isopropanol and were left for 24 hours at room temperature. By centrifugal evaporation, excess of ammonia and isopropanol were removed, the residue reconstituted in the mobile phase, filtered and spiked with a thiourea derivative as internal standard. For HPLC they used an RP-C18 column and the mobile phase was water with trifluoroacetic acid, where a linear gradient with methanol was adjusted.

Acid Hydrolysis Followed by SPE Extraction and Purification for HPLC Determination of Isothiocyanates

Bertelli *et al.* [141] reported a method for separation of sulforaphane by solid phase extraction with quantification by RP-HPLC. First the plant samples were grinded and homogenized with water, then hydrochloric acid was added (pH 3) to allow acid hydrolysis and the mixtures were left for 24 hours at room temperature. The analytes were extracted with dichloromethane and washed with water. The organic extracts were then purified by SPE with silicagel as adsorbent. The silica gel cartridges were first rinsed with dichloromethane, and then the sulforaphane extracts passed through the cartridge. Following, ethyl acetate was used to wash off the matrix and by rinsing with methanol, sulforaphane was recovered. By the use of HPLC consisting of a reversed phase C18 column and an UV/VIS detector, the content of sulforaphane was measured. A slight modification was used by Sivakumar *et al.* [131]. They first shock-froze plant material with liquid nitrogen and lyophilized the samples prior to grinding. The samples were then hydrolyzed with 0.1 M hydrochloric acid for 2 hours at 42 °C. In addition, the washing step of the dichloromethane extract was omitted. Because HPLC-UV detection of sulforaphane is insensitive due to the lack of a UV chromophore, Nakagawa *et al.* [142] made use of ELSD. ELSD is independent of UV properties as it is based on mass detection.

2.7.3 Methods for the Determination of Organic Acids

Organic acids naturally occur in fruits [143] and vegetables [144] and have a strong influence on organoleptic properties as flavor, color and aroma [145]. Further, as the organic acid profile is strongly related to the quality of vegetables and fruits, the nature and concentration of organic acids has been established as a chemical marker to assess the ripeness, quality, authenticity and the influence of agronomic factors as well as to distinguish different genotypes [146]. The predominant organic acids in vegetables and fruits are malic and citric acid [147] and are therefore mainly responsible for the product properties as taste, flavor and microbial stability

[148]. The role of citric acid in nature is very important as it is involved in the metabolic pathway (Krebs cycle) of the three basic nutrition types proteins, lipids and carbohydrates [149]. In addition, along with antioxidants it plays an important role in chelating trace metals and inhibiting enzyme activity [150, 151]. Following a summary of the methods known for the determination of organic acids, with a focus on citric acid, is given.

Analytical Methods for the Determination of Organic Acids in Food

Spectrophotometric, electrophoretic, chromatographic and enzymatic techniques have been applied for the determination of individual organic acids or acid profiles [152].

- Enzymatic techniques convince as methods for determination of individual organic acids due to their high sensitivity and selectivity. However, because each acid, for instance lactic, oxalic, acetic, malic and succinic acid, has to be determined separately, the study of organic acid profiles has the disadvantage of increasing costs and analysis time [153].
- As organic acids are non-volatile compounds, GC-MS/FID requires a derivatisation step [154, 155]. Normally the organic acids are transferred to their trimethylsilyl derivatives by using bis(trimethylsilyl)trifluoroacetamide and pyridine as derivatisation reagents [155]. The tedious sample preparation is in competition to the advantage of simultaneous determination of organic acids and high resolution.
- By using electrophoretic methods [148, 156], derivatisation of organic acids can be circumvented. High resolution together with simple sample preparation and shorter analysis time are the advantages of this technique, albeit on the cost of lower reproducibility.
- Due to the lack of sample derivatisation and high reproducibility, LC has prevailed against GC and electrophoretic methods. For the detection of the organic acids separated by LC, many different systems have been applied, like ultraviolet (UV) [143, 145, 155, 157–161], chemiluminescence [162], electrochemical methods [163, 164], conductivity [165], refractive index (RI) [158, 166] and mass spectrometry (MS) [167–169]. LC coupled to MS detection is considered to be the most selective and sensitive technique for the analysis of organic acids [169].
- Finally, also ion chromatography (IC) methods have been developed for the determination of organic acids. Normally IC is equipped with an ion suppressor and a conductivity detector. This has the advantage that interferences with, for instance, sugars or humectants are eliminated [170].

As mentioned above, LC is the most widely used method for the analysis of organic acids in fruits and vegetables. Common sample preparation procedures include either sample dilution followed by filtration or a more complex procedure, where organic solvent extraction and solid-phase extraction are used for cleanup. Additional workup is needed when organic acids

have to be analyzed in complex samples such as vegetables and fruits, as they contain a lot of interferences like phenols [146, 171]. However, in terms of precision and recoveries, solid phase extraction procedures lag behind the simpler sample treatments [172]. For analysis, reversed phase [146, 168, 169], ion exchange [165] and ion exclusion [155, 160, 161, 167, 171, 173] separation techniques are used, whereby ion exclusion is the most widely used technique. As detectors, UV-VIS and refractive index (RI) are traditionally used. Their simplicity is confronted with problems in terms of sensitivity and selectivity. By use of an MS detector, these problems can be circumvented. Following, two papers are represented exemplarily for the two types of sample preparations in combination with ion exclusion HPLC, as this is the most widely used technique.

Simple Sample Preparation with Ion Exclusion HPLC

A simple sample preparation followed by analysis via ion exclusion LC-MS/MS was published by Erro *et al.* [167]. The tandem MS system allows high sensitivity and selectivity together with a low demand on the purity of the sample.

- Sample extraction:
  Fresh samples were chopped in a mill with liquid nitrogen and were then stored at -80 °C till analysis. Prior to analysis, frozen samples were transferred into polypropylene tubes together with deionized water and an internal standard. The mixture was shaken on a vortex mixer, filtered and directly analyzed with a LC-MS/MS.
- Ion exclusion LC-MS/MS settings:
  For the separation of the organic acids, a "Rezex RHM-Monosachcaride H+" ion exclusion column was used together with an aqueous 0.1 % acetic acid mobile phase. The eluent flow rate was applied at 0.5 ml/min., the column oven temperature adjusted at 40 °C and the injection volume set at 30 µL. The analytes were ionized with a turboionspray operating in the negative ion mode and were detected with a "Q TRAP 3200" mass spectrometer.

As extractants, 100 % water, 10 % methanol in water and an aqueous 0.1 % acetic acid solution were tested. The results have shown that pure water is the most efficient extractant among this three.

Complex Sample Preparation with Ion Exclusion HPLC

A complex sample preparation, with the aim to remove interferences with solid phase extraction, together with ion exclusion HPLC-UV was reported by Valentao *et al.* [161].

- Sample extraction:
  Frozen pulverized samples were mixed with methanol on a waterbath at 40 °C. The extracts were filtered, methanol was removed under reduced pressure and the residue reconstituted in acid water (pH 2, HCl). The interfering matrix was then removed by passing the aqueous solution through an RP-C18 column, which was prior conditioned with methanol and acetic water. Polar compounds, such as organic acids, were eluted with an aqueous solution, while the nonpolar compounds were retained. Again the extract was concentrated to dryness and redissolved in the mobile phase.

- Ion exclusion HPLC-UV settings:
  For the separation of the organic acids, an ion exclusion column (Nucleogel Ion 300 OA) with a mobile phase, consisting of an aqueous 0.01 N sulfuric acid solution, was used. The column oven was set to 30 °C and the flow rate adjusted at 0.1 mL/min. As detector, a UV detector was set at 214 nm.

3 Materials and Methods

3.1 Methods

In this chapter, the fully developed methods for the determination of the enzymatic activity by UV/VIS spectrophotometry, of allyl isothiocyanate by GC-FID, of citric acid by LC-MS/MS as well as of sinigrin by LC-MS/MS are presented. All measurements of horseradish samples (see chapter 3.4) were carried out following these procedures. The development of these methods and their challenges will be discussed in chapter 4.

3.1.1 Determination of the Enzyme Activity of *Myrosinase*

Enzyme activity measurements were carried out using a "Agilent Technologie 60 UV-VIS" spectrophotometer equipped with a "Varian Single Cell Peltier Accessory Cary" peltier element. The isolation of the enzyme is based on a liquid-extraction procedure with water, including the steps of mechanical cell disruption, centrifugation and filtration. The determination of the enzymatic activity is closely related to the procedure of Li *et al.* [8] and evaluates the enzyme activity by the rate of hydrolysis of sinigrin, which correlates with the measurable value of the decline of the optical density.

Liquid Extraction of *Myrosinase*

3 g of the respective horseradish product were weighed into 50 mL PP-vials and 30 mL distilled water were added. In the case of horseradish roots, they were prior grated with a microplane grater. The samples where then put into an ice bath and homogenized with an UltraTurax at 9 500 rpm for 4 times each 1 minutes After each minute, the fibers stuck in the head of the UltraTurax were removed and brought back into the homogenate. Further, 10 mL of the homogenate were transferred to 15 mL PP-vials and centrifuged at 4 °C and 9 500 rpm (11 500 g) for 20 minutes The supernatant was then filter through PTFE filters (pore size 0.45 µm) and the extracts stored in the refrigerator till use.

Determination of the Enzyme Activity by UV/VIS Spectroscopy

For the determination of the enzymatic activity by UV/VIS spectrophotometry, a solution of 0.2 mM sinigrin in a 33.3 Molar potassium phosphate buffer was prepared. 2.870 mL of this solution and 130 µL of a 0.5 mM ascrobic acid solution were transferred into a quartz cuvette and pre-equilibrated to 37 °C for 3 minutes by the peltier element. The reaction was initiated by the addition of 100 µL of the extract. The decline in optical density, which results from the breakdown of sinigrin, was then monitored at 227 nm at 37 °C over a time period of 3 minutes.

3.1.2 Determination of Allyl Isothiocyanate by GC-FID

The measurement of allyl isothiocyanate was carried out using a gas chromatography - flame ionization detector (GC-FID) system from Hewlett Packard. The "HP 5890 Series II" was equipped with an "HP 6890 Series Injector" and a flame ionization detector. Extraction of allyl isothiocyanate was done by liquid extraction with dichloromethane as extraction solvent.

Liquid Extraction of Allyl Isothiocyanate

For the sample preparation of allyl isothiocyanate, 1 g of the respective horseradish products were weighed into glass screw-cap reagent tubes. To these samples, 5 mL of an extraction solution of 0.5 % triethylamine in dichloromethane and 100 µL of an internal standard solution of hexyl isothiocyanate with a concentration of 10 g/L were added. The extraction mixtures were then placed in an ultrasonic bath at room temperature for 30 minutes. Afterwards, the samples were centrifuged at 2400 rpm for 5 minutes. The organic phase was then transferred into 1.5 mL crimp-seal vials and analysed by GC-FID.

GC-FID Detection of Allyl Isothiocyanate

In order to develop a method for the determination of allyl isothiocyanate, to evaluate the pungency of horseradish products, an HP 5890 Series II system was employed. The signal of allyl isothiocyanate was plotted with a universal flame ionization detector. The samples from liquid extraction where transferred without dilution into 1.5 mL crimp-seal vials in order to feed the autosampler. As extraction solvent dichloromethane was applied, which caused massive corrosion of the detector. This problem was circumvented by the use of a Teflon inlet. Nevertheless, a preheating of the detector was necessary to prevent the condensation of water on the inlet. The identification of allyl isothiocyanate was achieved by comparison with a standard solution. In table 3.1 the analysis parameters of the allyl isotiocyanate method are depicted.

Table 3.1: Analysis parameters of the GC-FID method for allyl isothiocyanate

GC - FID Parameters	
Gas chromatograph	Hewlett Packard 5890 Series II
Detector	FID
Column parameters	
Column	ZB Wax (Zebron)
Length	30 m
Diameter	0.32 mm
Film thickness	0.25 µm
Min. temperature	40 °C
Max. temperature	250 °C
Carrier gas	Helium
Detector parameters	
Combustion gas	Syn. Air, Hydrogen
Aux gas	Nitrogen
Method parameters	
Injector temperature	220 °C
Detector temperature	240 °C
Injection volume	1 µL
Injection mode	Split
Split rate	15:01
Column inlet pressure	86 kPa
Temperature program	
90 °C (1 min) - 10 °C/min - 150 °C - 20 °C/min - 230 °C	

3.1.3 Determination of Citric and Isocitric Acid by LC-MS/MS

Citric and isocitric acid were analysed on a liquid chromatography - tandem mass spectrometry (LC-MS/MS) system from Shimadzu, equipped with a refrigerated autosampler, two pumping systems, allowing the appliance of gradients, a phenomenex kinetix column C18, a diode array detector and a triple quadrupole detector. As internal standard, tricarballylic acid was applied and all analytes were measured in the multiple reaction monitoring (MRM) mode. The extraction procedure was inspired by a liquid-extraction procedure published by Flores *et al.* [167], including the steps of homogenization, centrifugation and filtration. The choice of methanol and 0.3 % fromic acid in water as mobile phase was related to the work of Fernández-Fernández *et al.* [168].

Liquid Extraction of Citric and Isocitric Acid

First, the horseradish skin of the roots was removed with a potato peeler. Then roots were grated with a microplane grater and 2 g per sample weighed into 50 mL PP-Vials. Subsequent, 20 mL of methanol-water (1:1) as well as 200 µL of the internal standard solution (20 g/L tricarballylic acid in water) were added and the mixture homogenized with an UltraTurax twice (1 minutes + 0.5 minutes) at 13 500 rpm. Further, the samples were centrifuged at 3 000 rpm for 5 minutes and filtrated through a PTFE-filter (pore size 0.45 µm). After the samples were diluted 1 + 99, 1 + 499 and 1 + 999, the samples were analyzed by LC-MS/MS.

LC-MS/MS Detection of Citric and Isocitric Acid

The analysis of citric and isocitric acid was carried out on a "Shimadzu Nexera X_2" LC-MS/MS system under the analysis parameters shown in table 3.2 and 3.3. Citric, isocitric and tricarballylic acid were determined in the multiple reaction monitoring (MRM) mode, for which a quantifier ion and two qualifier ions each were detected. Retention of the analytes is based on the principle of ion pairing in presence of formic acid.

Table 3.2: MS parameters of the LC-MS/MS method for citric and isocitric acid

	Precursor m/z	Product m/z	Dwell time [msec]
	175.05	157.00®	41.0
Tricarballylic acid	175.05	87.05	41.0
	175.05	68.90	41.0
	191.05	111.00	41.0
Citric acid	191.05	87.05®	41.0
	191.05	84.90	41.0
	191.05	110.95	41.0
Isocitric acid	191.05	173.05	41.0
	191.05	73.00®	41.0
	Q1 pre bias [V]	CE	Q3 pre bias (V)
	12.0	14.0	29.0
Tricarballylic acid	18.0	17.0	15.0
	12.0	18.0	26.0
	20.0	13.0	20.0
Citric acid	21.0	17.0	15.0
	23.0	16.0	14.0
	21.0	13.0	19.0
Isocitric acid	13.0	11.0	17.0
	14.0	21.0	29.0

® indicates the product ions selected as quantifier ions

Table 3.3: Analysis parameters of the LC-MS/MS method for citric and isocitric acid

LC-MS/MS Parameters	
Liquid chromatograph	Shimadzu
Auto sampler	Nexera X_2 SIL - 30AC
Degasser unit	DGU - $20A_{5R}$
Pumps	Nexera X_2 LC - 30 AD
Column oven	CTO - 20 AC prominence
Diode array detector	Nexera X_2 SPD - M30A
Detector	LCMS - 8050
Communation Bus Modul	CBM - 20 A
Column parameters	
Column	C18 Phenomenex Kinetix
Length	100 mm
Diameter	2.1 mm
Particel size	1.7 µm
pH stability	1.5 - 10
Oven temperature	40 °C
Mobile phase and program	
Mobile phase A	0.3 \% (v/v) formic acid in water
Mobile phase B	methanol
Total flow	0.4 mL/min
Pumb B conc.	10 \%
Mode	isocratic
End time	3 min
Interface parameters	
Nebulizing gas flow	3 L/min
Heating gas flow	10 L/min
Interface temperature	300 °C
DL temperature	250 °C
Heating block temperature	400 °C
Drying gas flow	10 L/min
Mode	negative
MS parameters	
Interface voltage	-1 kV
Q1 Resolution	Unit
Q3 Resolution	Low
Event time	0.132 sec

3.1.4 Determination of Sinigrin by LC-MS/MS

Sinigrin was determined using a liquid chormatography - tandem mass spectrometry (LC-MS/MS) system from Shimadzu, equipped with a refrigerated autosampler, two pumping systems, allowing the appliance of gradients, a phenomenex kinetix column C18, a diode array detector and a triple quadrupole detector. Extraction was done by liquid-extraction according to an adapted procedure by Volden *et al.* [123] with water-methanol (1:1) as extraction solvent.

Liquid-Extraction of Sinigrin

Prior to extraction, horseradish roots were peeled with a potato peeler. Then the roots were grated with a microplane grater and as quickly as possible 1 g per sample were weighed into 50 mL PP-vials and immediately 20 mL of a boiling methanol-water mixture (1:1) added. In the case of grated horseradish products, the first two steps were not necessary. The methanol-water mixture had prior been heated on a stirring hot plate to 75 °C. The extraction mixture was then held at 75 °C under stirring in a heating block, in order to deactivate *myrosinase*. Following the samples were homogenized with an UltraTurax twice, each for 1 minute at 13 500 rpm. Further, the samples were centrifuged at 3 000 rpm for 10 minutes and filtered through a PTFE-filter (0.45 µm pore size). The extracts were then diluted 1 + 99 (1 µL injection volume) for roots and 1 + 9 (5 µL injection volume) for grated horseradish products, transferred to 1.5 mL screw-cap vials and analyzed by LC-MS/MS. As the density of the methanol-water mixture varies with the temperature, samples were weight before and after the addition of the extraction solvent and the volume was calculated from the density at 20 °C.

LC-MS/MS Detection of Sinigrin

The sinigrin method was developed on a "Shimadzu Nexera X_2" LC-MS/MS system. Sinigrin was identified by comparison with a standard solution and by its mass. For the determination of sinigrin, the triple quadrupole was operated in the multiple reaction monitoring (MRM) mode and a quantifier ion as well as two qualifier ions were appointed. In table 3.4 and 3.5 the parameters applied to the LC-MS/MS system are depicted. The choice of the column and the mobile phase were based on the research of Mellon *et al.* [122]. Retention of the polar compound sinigrin is achieved by ion pairing with trifluoroacetic acid.

Table 3.4: MS parameters of the LC-MS/MS method for sinigrin

	Precursor m/z	Product m/z	Pause time [msec]	Dwell time [msec]
	358.10	96.95⊛	3.0	130.0
Sinigrin	358.10	96.00	3,0	130.0
	358.10	75.00	3.0	130.0

⊛ indicates the product ions selected as quantifier ions

	Q1 pre bias [V]	CE [V]	Q3 pre bias [V]
	25.0	21.0	17.0
Sinigrin	25.0	38.0	17.0
	26.0	27.0	29.0

Table 3.5: Analysis parameters of the LC-MS/MS method for sinigrin

LC-MS/MS Parameters	
Liquid chromatograph	Shimadzu
Auto sampler	Nexera X_2 SIL - 30AC
Degasser unit	DGU - $20A_{5R}$
Pumps	Nexera X_2 LC - 30 AD
Column oven	CTO - 20 AC prominence
Diode array detector	Nexera X_2 SPD - M30A
Detector	LCMS - 8050
Communation Bus Modul	CBM - 20 A
Column parameters	
Column	C18 Phenomenex Kinetix
Length	100 mm
Diameter	2.1 mm
Particel size	1.7 µm
pH stability	1.5 - 10
Oven temperature	40 °C
Mobile phase and program	
Mobile phase A	0.1 \% (v/v) trifluoroacetic acid in water
Mobile phase B	methanol
Total flow	0.4 mL/min
Pumb B conc.	25 \%
Mode	isocratic
End time	3 min
Interface parameters	
Nebulizing gas flow	3 L/min
Heating gas flow	15 L/min
Interface temperature	300 °C
DL temperature	250 °C
Heating block temperature	400 °C
Drying gas flow	5 L/min
Mode	negative
MS parameters	
Interface voltage	-1 kV
Q1 Resolution	Low
Q3 Resolution	Unit
Event time	0.399 sec

3.1.5 Determination of Sinigrin by SFE-SFC-MS/MS

Determination of sinigrin by super critical fluid extraction (SFE) combined with super critical fluid chromatography - tandem mass spectrometry (SFC-MS/MS) was done on a "Nexera UC" SFE-SFC-MS/MS system from Shimadzu.

SFE-SFC-MS/MS Detection of Sinigrin

In table 3.6 and 3.7 the method parameters of the MS and interface optimization are shown. These parameters ware the basis for all further method development steps.

Table 3.6: Analysis parameters of the SFE-SFC-MS/MS method for sinigrin

LC-MS/MS Parameters	
Liquid chromatograph	Shimadzu
System	Nexera UC
Detector	LCMS - 8050
Interface parameters	
Nebulizing gas flow	3 L/min
Heating gas flow	15 L/min
Interface temperature	200 °C
DL temperature	250 °C
Heating block temperature	100 °C
Drying gas flow	5 L/min
Mode	negative
MS parameters	
Interface voltage	- 2.5 kV
Q1 Resolution	Unit
Q3 Resolution	Unit
Detector voltage	- 2 kV

Table 3.7: MS parameters of the SFE-SFC-MS/MS method for sinigrin

	Precursor m/z	Product m/z	Pause time [msec]	Dwell time [msec]
	358.20	97.20	3.0	6.0
Sinigrin	358.20	96.20	3.0	6.0
	358.20	75.25	3.0	6.0
	Q1 pre bias [V]	CE [V]	Q3 pre bias [V]	
	17.0	21.0	19.0	
Sinigrin	17.0	30.0	20.0	
	25.0	30.0	13.0	

3.2 Sample Preparation

In the list bellow, the equipment used for the sample preparation is depicted. Specific settings of the equipment will be elucidated at the respective passages in chapter 4.

Equipment	Supplier
UltraTurax	Heidolph DIAX 600
Ultrasonic Bath	Elma Transsonic T 780
pH-Meter	Hanna Instruments
pH-Meter	Metler Toledo Seven Compact
Cryo mill	Retsch Cryo Mill
Centrifuge (coolable)	Hettich Rotina 420 R
Centrifuge	Hermle Z 510
Lyophilisation	Christ Beta 2-8 LD_{plus}

3.3 Materials

Following materials were used in this thesis:

Material	Supplier	Description
PP-Vials	Sarstedt	30 mL
PP-Vials	Sarstedt	50 mL
PTFE-Filter	Macherey-Nagel Chromafil®xtra	pore size 45 µm, diameter 13 mm
Screw-cap vials	VWR	GL 18, 100x16 mm
Crimp-seal vials		1.5 mL
Screw-cap vials		1.5 mL
Quartz cuvette	Helmar Analytics	

3.4 Samples

All products were purchased from local supermarkets, except for root RB, which was delivered by our cooperation partner Feldbacher Fruit Partners. Fiber and paste like horseradish products were sold in transparent glasses with metal srew-caps. Roots were wrapped in plastic foil. All samples were stored in a cooling room at 7 °C. For the experiments it was carefully considered that the expiration date within brands was the same and that the difference between brands was as small as possible.

Type	Code	Description
Root	RA	green, white flesh, length 20 cm, diameter 3.5 - 2.0 cm
Root	RB	green, white flesh, length 22 - 26 cm, diameter 4.5 - 2.5 cm
Fiber	FA	95 % horseradish, 5 % rape seed oil, salt, sugar, citric acid, potassium metabisulfite
Fiber	FB	95 % horseradish, rape seed oil, salt, citric acid, sodium metabisulfite
Fiber	FC	94.5 % horseradish, 4.5 % rape seed oil, salt, citric acid, sodium metabisulfite
Fiber	FD	95 % horseradish, rape seed oil, salt, sugar, cE330, sodium metabisulfite
Fiber	FE	95 % horseradish, rape seed oil, salt, citric acid, sodium metabisulfite
Paste	PF	65 % horseradish, water, sunflower oil, distilled vinegar, citric acid, E224, Na-saccharide
Paste	PF-Obers	24 % horseradish, water, sunflower oil, 12 % cream (15 % fat), distilled vinegar, sugar, modified starch, milk powder, whey protein, salt, xanthan, guar gum, citric acid, ascorbic acid, E224, K-sorbate, Na-benzoate
Paste	PF-Apple	18 % horseradish, 77 % apple, apple vinegar, Na-saccharide, E224, ascorbic acid
Paste	PC-Mustard	21 % horseradish, water, rape seed oil, 70 % mustard seeds, distilled vinegar, sugar, milk powder, whey protein, salt, xanthan, guar gum, citric acid, ascorbic acid, E224, K-sorbate, Na-benzoate, natural aroma, spices

4 Experimental

4.1 Method Development: Allyl Isothiocyanate by GC-FID

4.1.1 Calibration of Allyl Isothiocyanate

For the calibration of allyl isothiocyanate the calibration standards were prepared in dichloromethane. The selected concentrations were 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/g, whereby the volume of the calibrations standards of 1 mL corresponded to 0.2 g of horseradish samples. As standard solution a 1 g/L solution of allyl isothiocyanate in methanol was used. To each calibration point, which were prepared in 3 replicates, 20 µL of a 10 g/L standard solution of hexyl isothiocyanate were added. The analysis of the calibration points was performed with the GC-FID method described in chapter 3.1.2.

4.1.2 UltraTurax Homogenization vs. Ultrasonic Liquid Extraction.

In the beginning of the method development for the determination of allyl isothiocyanate, two liquid extraction procedures were tested on their suitability for the extraction of allyl isothiocyanates from horseradish products. Both were quite similar and differed only in the way the extraction was assisted. One was assisted by sonification and the other extraction procedure was assisted by a homogenization with an UltraTurax.

UltraTurax assisted liquid extraction
1 g per sample were weighed into 50 mL PP-vials and 5 mL of dichloromethane and 100 µL of hexyl isothiocyanate (10 g/L) as internal standard were added. Then the samples were homogenized with an Ultra Turax for 1 minutes, centrifuged at 2 400 rpm for 5 minutes and the supernatants transferred into 1.5 mL crimp-seal vials. The extracts were analyzed by GC-FID according to the procedure described in chapter 3.1.2.

Ultrasonic assisted liquid extraction
1 g per sample were weighed into 15 mL PP-vials and 5 mL of dichloromethane and 100 µL of hexyl isothiocyanate (10 g/L) as internal standard were added. Then the samples were sonificated for 30 minutes, centrifuged at 2 400 rpm for 5 minutes and the supernatants transferred into 1.5 mL crimp-seal vials. The extracts were analyzed by GC-FID according to the procedure described in chapter 3.1.2.

In order to test these two extraction procedures, two grated horseradish products (fibers) and two paste like horseradish products (pastes) were analyzed in 3 replicates each. As the results were unsatisfactory, different tests were carried out in order to determine the parameters influencing the results of the two liquid extraction procedures. All tests used the same two grated horseradish products and the same two paste like horseradish products.

Test 1 As allyl isothiocyanate is hydrolytically very unstable, the UltraTurax procedure was repeated without the use of water. Instead, 2 g per sample with 10 mL dichloromethane were used for extraction. Water was initially added in order to increase the volume of the solvent, as a distinct volume is needed for the homogenization with an UltraTurax.

Test 2 Whether the time of homogenization with the UltraTurax had an influence on the results was tested by altering the homogenization time from 0.5 to 1, 1.5 and 2 min.

Test 3 Horseradish products contain a lot of water, therefore Na_2SO_4 was added to dry the organic phases of both extraction procedures in order to evaluate whether the humidity of the sample itself is high enough to cause strong hydrolysis of allyl isothiocyanate.

Test 4 In order to check whether their is an influence of the solvent on the results, besides dichloromethane also toluene, ethyl acetate and a mixture of dichloromethane with pentane 1:1 were tested as alternative extraction solvents.

4.1.3 Optimization of the Sonification Assisted Liquid Extraction Procedure

The sonification assisted liquid extraction procedure showed more reliable results than the UltraTurax procedure. Therefore the sonification assisted extraction was singled out and was further optimized.

Standard Addition

In order to get a feeling for the expected values of allyl isothiocyanate in the products and to minimize matrix and instrumental effects, a standard addition procedure was performed. For the standard addition, a grated (Fiber 1) as well as a paste (Paste 1) like horseradish product were used. For each sample, six spiking concentrations were prepared, whereby each concentration step was performed in three replicates. The spike concentrations were 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/g. The liquid extraction assisted with sonification was performed according to the procedure described in chapter 4.1.2.

Stability Test

With the aim to identify the source of the great variations of the analyte values of the standard deviation, a stability test was performed. For this matter, 10 horseradish simulants were

prepared. Each consisted of 5 mL dichloromethane, 1 mg/g allyl isothiocyanate and 1 mg/g hexyl isothiocyanate, whereby 5 mL were equal to 1 g of a horseradish sample. The horseradish simulants were analyzed according to the GC-FID method described in chapter 3.1.2. The simulants were injected fife times each and this set of 50 measurements was repeated 4 times at different injector temperatures of 180, 200, 220 and 240 °C. This provided a total a measurement time of about 71 hours.

Displacer

The stability of the analyte and internal standard could be proven, so the next aspect which was tested was the influence of the matrix. Triethylamine as a displacer was used as an attempt to overcome matrix effects. For the test the known sample setup of grated as well as paste like horseradish products was tested. In contrast to the extraction procedure described in chapter 4.1.2, triethylamine was added to the extraction solvent. As the amount of displacer plays a major role, extractions were performed with 0.1, 0.25, 0.5 and 1.0 % triethylamine in dichloromethane. Each sample was prepared in 3 replicates.

Method check

Due to the great improvement of the method by the use of triethylamine as displacer, the method developed so far was tested for two different brands of grated horseradish and four different brands of paste like horseradish products. From each brand one sample was prepared three times. Therefore 1 g of the respective sample were weighed into 15 mL glass vials, as a test revealed that the use of glass vials instead of PP-vials leads to better results. This might mainly be due to the tighter closing of the caps of the glass vials, which contained a septum. To the samples, further 5 ml of a mixture of 0.5 % tiethylamine in dichloromethane was added and 100 µL of a 10 g/L standard solution of hexyl isothiocyanate in methanol. The analysis of the extracts by GC-FID was done according to the procedure described in chapter 3.1.2.

Determination of the pH of six horseradish products

As vinegar is a common additive in horseradish products (cf. chapter 3.4), especially in paste like products, a different pH compared to the other samples could be the reason for the fluctuations of the internal standard in the case of sample PF. Because of that, the pH was determined for the six different horseradish products. 2 g per sample were weighed into 50 ml PP-vials and 10 mL of deionized water were added. The samples were stirred with a stirring bar on a magnetic stirrer for 10 minutes and subsequently the pH was measured with a pH glass electrode.

Homogeneity of grated horseradish products

To find out whether a lack in homogeneity of sample FA caused the great variation of the values, sample FA was transferred into a 500 mL glass beaker and intensively blended with a spatula. Then three replicates were prepared and analyzed according to the procedure described in chapter 3.1.2.

4.1.4 Validation of the Allyl Isothiocyanate Method

With the aim to evaluate the suitability of the developed method, a method validation was carried out with the assistance of "ValiData" (Excel-Makro zur Methodenvalidierung, Univ.-Prof. Wolfhard Wegscheider). The validation was performed based on a standard addition procedure. For the simulation of the matrix, 25 g of horseradish fibers (sample FB) were extracted with 122.5 mL of a mixture of 0.5 % triethylamine in dichloromethane. After 2.5 mL of the internal standard (10 g/L in methanol) were added, the extracts were sonificated for 30 minutes. In order to minimize the loss of dichloromethane, the extraction was performed in a cooling chamber and the sonification bath was cooled with ice. The organic phase was divided into 50 mL PP-vials in 30 mL proportions prior to centrifugation at 2400 rpm for 5 minutes. As spiking concentration steps 0, 0.3, 0.6, 0.9, 1.2 and 1.5 mg/g horseradish sample were selected. Each concentration step was done in three replicates. A standard consisted of 1 000 µL of horseradish extract, which is representative for 0.2 g of a horseradish sample, and 20 µL of an appropriate spike concentration of the analyte. Analysis of the standards was performed with GC-FID, according to the procedure described in chapter 3.1.2.

4.1.5 Depth Profiles of Allyl Isothiocyanate in Grated Horseradish Products

So far, the inhomogeneous distribution of allyl isothiocyanate was proven for one grated horseradish sample. In order to evaluate whether this inhomogeneity is common for all brands selling grated horseradish products and whether there are differences between and within the brands, depth profiles for fife brands encoded as FA, FB, FC, FD, FE (cf. chapter 3.4) and for each brand two samples were analyzed according to the procedure described in chapter 3.1.2. For the depth profiles, the sample glasses were separated into 6 layers of distances between 1 and 1.2 cm and into a right and left side to increase the amount of sample points (see figure 4.1). In total 12 sample points were analyzed per sample. Additionally, each sample was homogenized by blending in a 500 mL glass beaker and three replicates were analyzed.

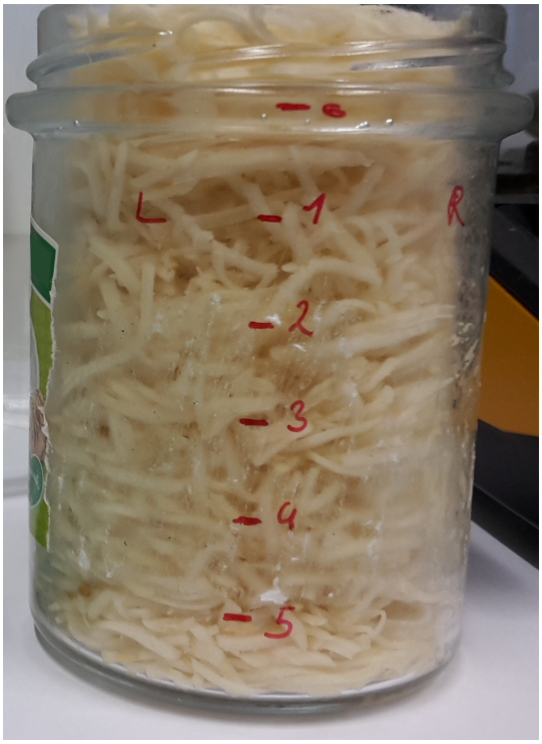


Figure 4.1: A depth profile consisted of 12 sample points per sample. Therefore the sample glasses were divided into six layers with distances of 1 to 1.2 cm and into a left and right side.

4.1.6 Evaluation of the Decrease in Pungency of Two Different Brands

The purpose of the next experiment was to evaluate whether the product of brand FA shows a stronger decrease in pungency over five days than the product of brand FB. The optimized production of brand FB leads to the assumption that its product undergoes a smaller loss of pungency than the competitor. For each brand, fife samples of a grated horseradish product were bought in local supermarkets. Samples were analyzed five times on five consecutive days. For each sample and day, three replicates were prepared. In order to simulate the use of a consumer, the samples were taken from the top. After sampling, the samples were held at room temperature for a distinctive period of time before they were stored again in the cooling chamber at 7 °C. Each day the exposure time at room temperature was altered, starting from 30 to 60, 120 and 300 min. To guarantee a reproducible workflow, the samples were transported in a cold box with ice packs. After a sample was weighed into a glass vial, a second person immediately added the extraction solvent, whereupon the samples could be assumed as "safe". Further, the samples of the brands were prepared in an alternating way.

4.2 Method Development: Enzyme Activity Determined by UV/VIS Spectroscopy

4.2.1 Isolation of *Myrosinase*

At the beginning of the method development for the isolation of *myrosinase*, 3 g of grated horseradish products were weighed into 50 mL PP-vials and 30 mL distilled water were added. The samples where then homogenized with an UltraTurax at 9500 rpm four times, each for 1 minute. Further, the homogenates were centrifuged at room temperature and 3 400 rpm for 30 minutes. Then aliquotes of the supernatant were filter through a PTFE and a cellulose filter and the extracts with an additional unfiltered extract stored in the refrigerator till use.
As no enzyme activity could be measured for grated horseradish products, the extraction was repeated at lower temperatures. It was thought that the enzyme could be destroyed in the centrifuge, which developed rather hot temperatures. Therefore the homogenization was done in an ice bath and the extracts centrifuged at 9 500 rpm for 20 minutes at 4 °C.
As still no enzyme activity was found, the procedure was repeated with horseradish roots, which were peeled prior to homogenization and grated with a microplane grater.

4.2.2 Enzyme Activity Determination

The enzymatic activity was first determined according to the procedure published by Li *et al.* [174]. For this matter, a mixture of 0.2 mM sinigrin in a 33.3 Molar potassium phosphate buffer was prepared. 3 mL of this solution were transferred into a quartz cuvette and pre-equilibrated to 37 °C for 3 minutes by a peltier element. The reaction was initiated by the addition of 100 µL of the extract. The decline in optical density, which results from the breakdown of sinigrin, was then monitored at 227 nm at 37 °C over a time period of 3 minutes.
According to Burmeister *et al.* [82], ascorbate is a cofactor for *myrosinase*. Taking this fact into account, the procedure above was adapted. Instead of 3 mL of the mixture of sinigrin in a potassium phosphate buffer, 2.870 mL were mixed with 130 µL of a 0.25 mM ascorbic acid solution in water in a quartz cuvette prior to initiation of the reaction by the addition of 100 µL of the extract.

4.2.3 Calculation of the Enzyme Activity

The enzyme activity (in units) was calculated according to the equation 4.1 published by Li *et al.* [174]. ΔA/Δt corresponds to the slope of the decline in optical density.

$$\frac{units}{cm^3} = \frac{\Delta A}{\Delta t} * \frac{1}{\epsilon l} * \frac{V_A}{V_E} * 10^3 \tag{4.1}$$

Δ t... reaction time in minutes (3 min.)

ϵ... extinction coefficient of sinigrin a 227 nm is 6784 $M^{-1}cm^{-1}$ [174]

l... cuvette path length (1 cm)

V_A... total volume of the assay mixture (3 mL)

V_E... volume of the enzyme solution (100 µL)

One unit of activity is defined as the amount of *myrosinase* that catalyzes the hydrolysis of 1 µmol of substrate per minute.

4.2.4 Dependence of the Enzyme Activity on the Ascorbate Concentration

In order to evaluate the influence of the ascorbate concentration on the enzyme activity, an experiment was designed using the developed method described in chapter 3.1.1, whereby the ascorbate conncentrations were altered. The added volume of ascorbate was 130 µL. The concentrations and the corresponding standard solutions are shown in table 4.2 .

Table 4.2: Depicted are the ascorbate concentrations in the reaction mixture as standard solutions from which 130 µL were added to the reaction mixture.

End conc. [mM]	End conc. [mg/L]	mg in 3 mL	Standard solution [mg/L]
0.125	22	0.066	0.51
0.250	44	0.132	1.02
0.500	88	0.264	2.04
0.750	132	0.396	3.06
1.000	176	0.528	4.08

4.3 Method Development: Citric and Isocitric Acid by LC-MS/MS

For the method development of citric and isocitric acid with tricarballylic acid as internal standard, standard solutions of 1 g/L were prepared in methanol for each analyte. As isocitric acid was not soluble in pure methanol, prior to the addition of methanol, isocitric acid was dissolved in some droplets of water. As mobile phase A 0.3 % (v/v) of formic acid in water and as mobile phase B methanol were employed.

Determination of precursor and product ions

Normally analyte concentrations of 1 mg/L are used for the method development, however in the case of these three acids, standard solutions had to be diluted to a concentration of 10 mg/L in a mixture of MeOH:H_2O=1:1 in order to receive strong enough signals. For the determination of the precursor and product ions, the LC-MS/MS was operated under the standard conditions recommended by Shimadzu in the negative ion mode, as this mode showed higher intensities compared to the positive ion mode. As precursor ions the selected m/z are 191.05 for citric and isocitric acid and 175.05 for tricarballylic acid. Based on a product ion scan, the product ions were selected according to their intensities, m/z 111.00, 87.05 and 84.90 for citric acid, 110.95, 173.05 and 73.00 for isocitric acid and 157.00, 87.05 and 68.90 for tricarballylic acid. With the chosen ions, multiple reaction monitoring (MRM) events were generated and the voltages applied to the quadrupoles were optimized. In order to select the quantifier ions, different concentrations of mixtures of all three analytes were prepared, whereby the concentration of one analyte varied while the other two were constant. By comparison of the results the selectivity of the MRM events was evaluated, which was especially necessary for citric and isocitric acid with the same precursor ion m/z.

Optimization of the separation

For the separation of the analytes, a reversed phase C18 Phenomenex Kinetix column was used. The mechanism responsible for the separation is based on the ion-pairing of acids with protons at a low pH. For this matter, the mobile phases water and methanol were acidified with formic acid. The retention behavior was studied with 0.1 and 0.3 % of formic acid in the mobile phases. Additionally, isocratic as well as programmed mobile phase conditions were tested.

Optimization of the electrospray ionization source (ESI)

After the separation conditions had been optimized, the ratio between heating and drying gas, the interface voltage, the interface temperature, the desolvation line (DL) temperature, the heating block temperature and the resolution modes of the quadrupoles were varied, whereby

temperatures were increased and decreased by 20 °C from standard conditions and interface voltages were altered from -1 to -5.

4.3.1 Calibration of Citric and Isocitric Acid

The calibration was performed in MeOH:H_2O=1:1 as solvent. As calibration points, the concentrations 0.1, 0.5, 1, 2, 5, 10 with each 2 mg/L of the internal standard were selected. 1 g/L standard solutions of analytes in methanol were used and diluted to 100 mg/L and 10 mg/L in the case of citric and isocitric acid and for the internal standard, tricarballylic acid was diluted to 200 mg/L in MeOH:H_2O=1:1 for the preparation of the calibration points. The determination of the calibration points was performed with the optimized method summarized in chapter 3.1.3. As the calibration points 5 and 10 mg/L showed an ion suppression, the concentrations 1, 2, 3, 4 and 5 mg/L were tested to find out which concentrations are affected by an ion suppression. As the ion suppression starts significantly at a concentration of 4 mg/L, the calibration was repeated for the concentrations 0.1, 0.25, 0.5, 0.75, 1, 2 and 3, whereby each calibration point was prepared in three replicates.

4.3.2 Validation of the Citric and Isocitric Acid Method

With the purpose to evaluate the suitability of the developed method, a method validation was carried out with the assistance of "ValiData" (Excel-Makro zur Methodenvalidierung, Univ.-Prof. Wolfhard Wegscheider). The validation was performed similarly to a standard addition procedure. For the simulation of the matrix, an extract of horseradish roots was prepared according to the procedure described in chapter 3.1.3. The extract was diluted 1 + 999 in order to fit into the calibration curve. Each sample consisted of 1 000 µL of the horseradish extract and 20 µL of the respective spike concentration. According to the assumption that horseradish roots contain approximately 6 g/kg citric acid, the spike concentrations selected were 0, 2, 4, 6, 8, and 10 g/kg citric acid. The corresponding standard solutions of citric acid were 0, 10, 20, 30, 40 and 50 mg/L and were prepared in water. Each spike concentration was performed in three replicates.

4.3.3 Amount and Distribution of Citric Acid in Horseradish Roots

With the developed method, the amount and distribution of citric acid should be determined as well as a sample preparation procedure should be tested. The sample preparation procedure included the following steps: First, two horseradish roots were peeled with a potato peeler and cut into four (RB1) and five (RB2) pieces, respectively. The pieces were additionally divided into an inner and an outer part (cf. figure 4.2). Each of the samples, eight for RB1 and ten for RB2, was separately grated with a microplane grater. Following, 2 g per sample were weighed

into 50 mL PP-vials and 20 mL of MeOH:H_2O=1:1 as extraction solvent and 200 µL of the internal standard (tricarballylic acid, 20 g/L in water) were added. The samples were then homogenized with an UltraTurax at 13 500 rpm each sample twice (1 minute + 0.5 minutes). The best homogenization results were achieved, when the samples were grated across the grain. Then samples were centrifuged at 3 000 rpm for 5 minutes, followed by filtration through a PTFE filter (0.45 µm pore size) and dilution with MeOH:H_2O=1:1 1 + 99, 1 + 499 and 1 + 999, according to the small working range of the calibration curve.

Figure 4.2: Shown is sample RB1 before and after the root was peeled. In order to generate a profile of the distribution of citric acid in horseradish roots, root RA1 was divided into 4 parts in the longitudinal direction. Each part was divided into an inner and outer part.

4.4 Method Development: Glucosinolates by LC-MS/MS

For the method development of the determination sinigrin, a standard solution of 1 g/L in water was prepared and as mobile phase A water with 0.1 % trifluoroacetic acid and as mobile phase B methanol with 0.1 % trifluoroacetic acid were employed.

Determination of precursor and product ions

Again, the generally used 1 mg/L concentration of the respective analyte was too low for the precursor ion search. By injection of 1 µL of a 10 mg/L sinigrin solution in MeOH:H_2O=1:1 it was possible to identify the m/z of 358.10 as the precursor ion of sinigrin, whose intensity was the highest in the negative mode. As product ions the identified m/z values were 96.95, 96.00 and 75.00. The highest intensity was found for m/z of 96.95, whereupon this product ion was selected as the quantifier ion. The others were used as qualifier ions. Multiple reaction monitoring (MRM) events were generated and the voltages applied to the quadrupoles were optimized.

Optimization of the separation

The separation was performed on a reversed phase C18 Phenomenex Kinetix column. Separation was achieved by an ion-pairing mechanism in presence of trifluoroacetic acid. According to Mellon *et al.* [122] 0.1 % trifluoroacetic acid in water and methanol were applied as mobile phases. Programmed as well as isocratic mobile phase conditions with different ratios of mobile phase A and B were tested in order to achieve a symmetrical peak form. Due to the lack of other glucosinolate standards, an optimization of the separation was not necessary , or rather not possible.

Optimization of the electrospray ionization source (ESI)

After the separation conditions had been optimized, the ratio between heating and drying gas, the interface voltage, the interface temperature, the desolvation line (DL) temperature, the heating block temperature and the resolution modes of the quadrupoles were varied, whereby temperatures were increased and decreased by 20 °C from standard conditions and interface voltages were altered from -1 to -5.

4.4.1 Calibration of Sinigrin

For the calibration of sinigrin MeOH:H_2O=1:1 was chosen as solvent system and the selected calibration points were 0.05, 0.1, 0.5, 1, 2, 5 and 10 mg/L of sinigrin, whereby each concentration was prepared in three replicates. A 1 g/L standard solution of sinigrin in water was used

and diluted to 10 and 100 mg/L in MeOH:H_2O=1:1 for the preparation of the calibration points. The determination of the calibration points was performed with the optimized method summarized in chapter 3.1.4.

4.4.2 Validation of the Sinigrin Method

In order to evaluate the suitability of the developed method, a method validation was carried out with the assistance of "ValiData" (Excel-Makro zur Methodenvalidierung, Univ.-Prof. Wolfhard Wegscheider). The validation procedure was performed based on a standard addition procedure. For the simulation of the matrix, an extract of horseradish roots was prepared according to the procedure described in chapter 3.1.4. The extract was diluted 1 + 99 in order to fit into the calibration curve. Each standard addition point consisted of 1 000 µL of the horseradish extract and 20 µL of the respective spike concentration. Preliminary studies revealed concentrations of sinigrin in horseradish roots of around 4 mg/g, according to that the selected spike concentrations were 0, 2, 4, 6, 8 and 10 mg/g sinigrin. The corresponding standard solutions of sinigrin were 0.05, 0.1 , 0.15, 0.20 and 0.25 mg/mL and were prepared in water. Each concentration step was performed in three replicates.

4.4.3 Amount and Distribution of Sinigrin in Roots and Processed Horseradish

Based on the optimized method, a sample preparation procedure was developed. For the extraction of sinigrin a liquid-extraction procedure was chosen, with MeOH:H_2O=1:1 as extraction solvent. The main elements of this procedure were inspired by Volden *et al.* [123] and adapted to the issue of horseradish roots and fiber products. The crucial point in the sample preparation was the inactivation of *myrosinase*, in order to prevent the loss of glucosinolates by their degradation. In the first step of the sample preparation procedure horseradish roots were peeled. Then each sample was grated separately with a microplane grater and as quickly as possible 1 g per sample was weighed into 50 mL PP-vials and immediately 20 mL of a boiling methanol-water mixtrue (1:1) was added (75 °C). In the case of grated horseradish products the first two steps were unnecessary. After a root sample was grated the time until the boiling methanol-water mixture is added has to be kept as short as possible to keep the loss of glucosinolates by hydrolysis as small as possible. After this, the samples were held at 75 °C under stirring in a heating block in order to achieve a complete deactivation of *myrosinase*. Following, the samples were homogenized with an UltraTurax twice for one minute each at 13 500 rpm. Then the samples were centrifuged at 3 000 rpm for 10 minutes and filtered through a PTFE-filter (0.45 µm pore size). According to the values found in literature for sinigrin in horseradish roots, which varies from 11.8 to 1.8 mg/g of horseradish roots, samples were diluted 1 + 99 and 1 + 9 prior to analysis by LC-MS/MS. As the volume of

liquids varies with the temperature, the samples were additionally weighed before and after the addition of the boiling methanol-water mixture. From the difference in weight and the density of the methanol-water mixture of 0.9156 g/mL at 20 °C [175] the added volume was calculated. The sample preparation procedure was tested for grated horseradish products as well as for roots. In order to ensure the complete inactivation of *myrosinase*, the sample preparation procedure was repeated with altering times of the samples in the heating block at 75 °C. Grated products were tested at 15, 30 and 45 minutes and root samples at 15 and 30 minutes.

Based on the fully developed method (see chapter 3.1.4), the distribution of sinigrin in two horseradish roots (RB3, RB4) as well as the profile of sinigrin in two grated horseradish products was determined. For this matter, roots were divided into 4 parts in the longitudinal direction and each part into an inner and outer part (cf. figure 4.2). In the case of grated horseradish products, the glass jars were divided into six layers in distances of approximately 1 cm (cf. figure 4.1). According to the preliminary tests of the sample preparation procedure, the time for the inactivation of *myrosinase* at 75 °C was set to 15 minutes.

4.4.4 SFE-SFC-MS/MS

During a four-day-stay in Duisburg at the European headquarter of Shimadzu the aim was to develop a method for the determination of sinigrin with SFE-SFC-MS/MS. For the development of the parameters of the MS and the electrospray ionization source, a 1 g/L standard solution of sinigrin was prepared in water. For the qualitative and quantitative determination of sinigrin one quantifier and two qualifier ions were selected from a precursor ion in MRM mode. The corresponding parameters are shown in chapter 3.1.5.

In order to optimize the extraction as well as separation parameters, the samples were specifically prepared for the SFE-SFC. The roots and grated horseradish products were prepared in five different ways. Slices of peeled roots or grated horseradish products were:

- frozen in liquid nitrogen and lyophilized.
- ground in a cryomill and lyophilized.
- ground in a cryomill and dried in an oven.
- dried in a vacuum oven.
- dried in an oven.

The aim of these preparation procedures was to inhibit *myrosinase* and to receive samples which can easily be crushed. Dried samples are further needed in order to perform SFE. The samples prepared at the university were transported in 50 mL PP-vials. The following separation parameters were tested to optimize the extraction and separation:

Column:	Ethylpyridine, C18 Intersil
Co-solvent:	MeOH, MeOH + 0.1 % trifluoroacetic acid, H_2O:MeOH=1:1 + 0.1 % trifluoroacetic acid
Extraction:	CO_2, CO_2 + 10 % H_2O, CO_2 + 10 % H_2O with 0.1 % trifluoroacetic acid
Split:	Backpressure A = 14 , B = 15 MPa; A = 15 , B = 40 MPa
T. extraction:	40 °C, 50 °C, 60 °C, 70 °C

5 Results and Discussion

5.1 Method development GC-FID Allyl Isothiocyanate

5.1.1 Calibration of Allyl Isothiocyanate

The calibration of allyl isothiocyanate revealed a linear behavior with a coefficient of determination of 0.9986. The low values of the slope and intercept are caused by plotting the signalratio of allyl isothiocyanate to the internal standard against the calibration concentrations. The prepared three replicates per calibration standard had in average a standard deviation of 1.5 % and the twofold injections showed reproducibilities of 1.0 % in average. Calibration of the LOD and LOQ, based on the calibration method, revealed values of 0.04 mg/g and 0.13 mg/g, respectively.

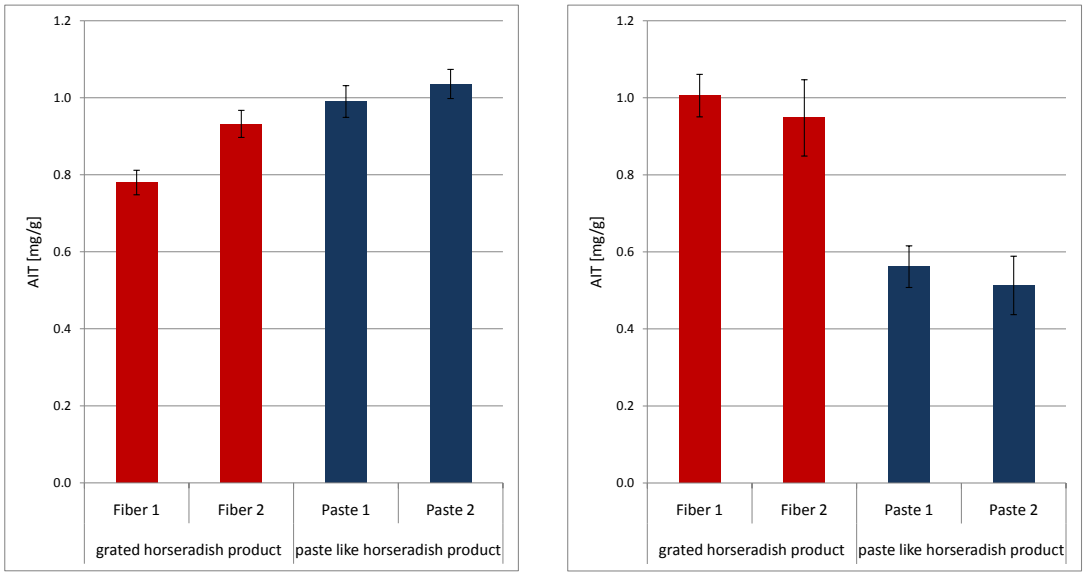
Table 5.1: Key parameters of the allyl isothiocyanate calibration

	Slope	Intercept	R2	LOD[®] [mg/L]	LOQ[®] [mg/L]
Sinigrin	0.8338	0.0072	0.9986	0.04	0.13

[®] LOD and LOQ were calculated based on the calibration method.

5.1.2 UltraTurax Homogenization vs. Ultrasonic Assisted Liquid Extraction.

The results of the test for the evaluation of the suitability of the two liquid extraction procedures, one assisted by sonification and one by an UltraTurax (see figure 5.1), revealed that in the case of the sonification procedure, the grated horseradish products (fiber 1 and 2) showed higher values of allyl isothiocyanate than pastes (paste 1 and 2). This is compatible with the fact that horseradish fibers contribute 95 % of the grated product content and 65 % of the content of paste like products. However, the data had a rather miserable reproducibility of the analyte of 8.0 ± 5.0 % and a recovery of the internal standard of 96.0 ± 11.0 %. In comparison fibers and pastes of the UltraTurax procedure showed comparable amounts of allyl isothiocyanate, where the values of the fibers were a bit lower. But in this case the data had a good reproducibility for the analyte of 3.0 ± 1.6 % and also a good recovery of the internal standard of 103.0 ± 1.6 %.



(a) UltraTurax assisted extraction

(b) Sonification assisted extraction

Figure 5.1: Test results of the evaluation of the suitability of the UltraTurax and sonification assisted liquid extraction of allyl isothiocyanate from grated and paste like horseradish products.

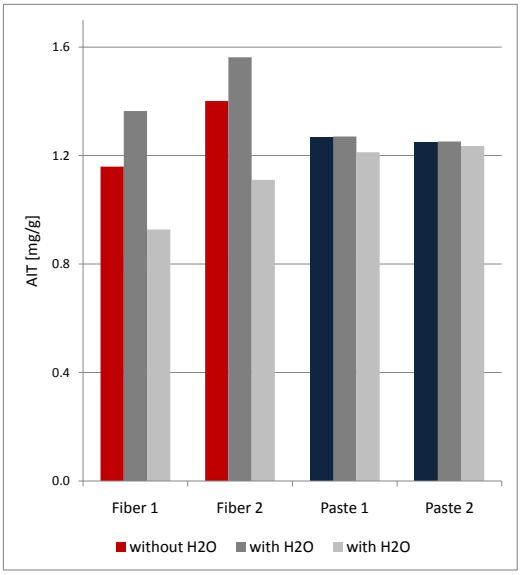
The first results were not satisfactory as the sonification procedure suffered from rather strong fluctuations of the replicates and the UltraTurax procedure showed implausible values. Therefore four different tests were carried out in order to identify the parameters which might influence the results.

Test 1 According to the hydrolytically unstable allyl isothiocyanate, the UltraTurax extraction procedure was carried out once without water and twice with water. Initially water was added to increase the solvent volume, as the UltraTurax needs a minimum volume to grease the Teflon bit. Water was used, because it is immiscible with dichloromethane. In figure 5.2a the results of the test are represented. In case of the paste like products, water showed no influence at all. For grated horseradish products, the two preparations with water showed strongly varying results. This leads to the conclusion that there is an influence of water or it is a problem of the product homogeneity. If these were the case, the mean values of the extractions using water would be approximately of the same amount as the extraction without water.

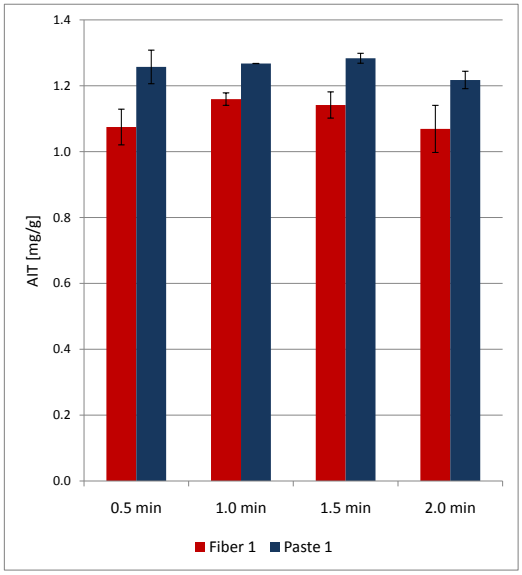
Test 2 In the second test the influence of the time of homogenization with the UltraTurax was examined. As the results in figure 5.2b show, the time of homogenization had no effect on the results. Actually, by using an UltraTurax extraction, solvent was lost by evaporation. The lost volume increased with the time of homogenization. However, this effect is compensated by the internal standard.

Test 3 Horseradish products, especially paste like products, have a high water content. In order to eliminate also this source of hydrolysation, Na_2SO_4 was added to the extraction mixture. As the results in figure 5.2c show, the use of Na_2SO_4 had no effect on the results of the UltraTurax extraction and in the case of the sonification procedure it led to an adjustment of the values of the fiber and paste products.

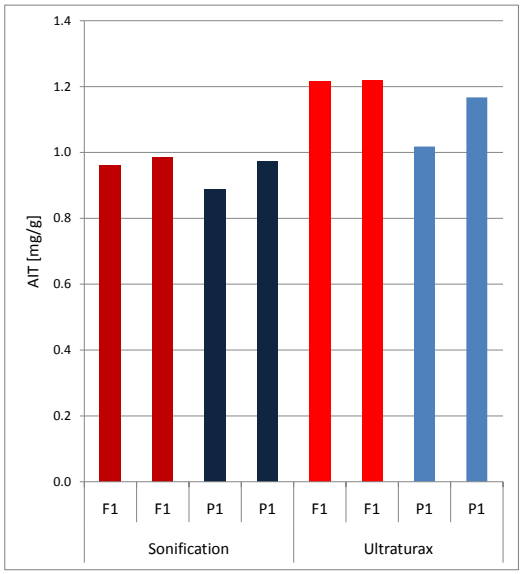
Test 4 The examination of the influence of the solvent type on the extraction results revealed that there is no influence in the case of the UltraTurax assisted extraction procedure (see figure 5.2d). For the sonification procedure (see figure 5.2e) the results show that except for the mixture of dichloromethane with pentane, all other solvents provided higher values for grated than for paste like horseradish products. However, dichloromethane showed the highest extraction efficiency and was therefore maintained as extraction solvent.



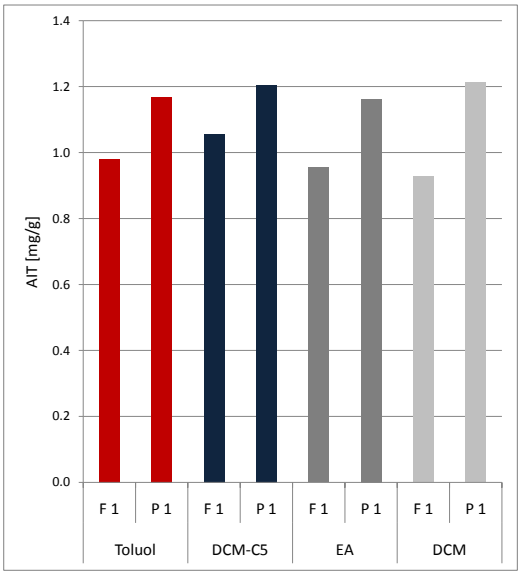
(a) Water



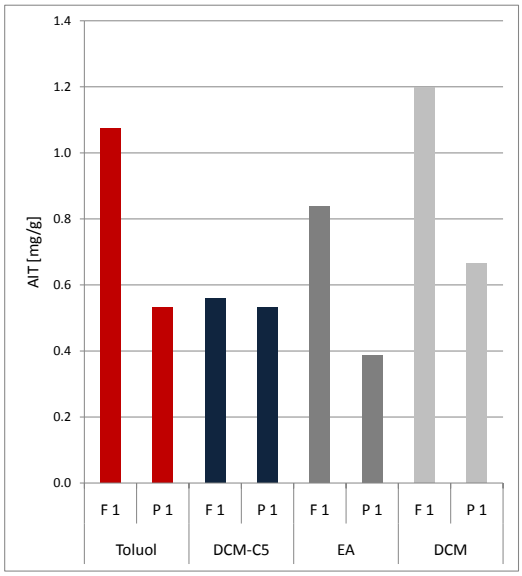
(b) Duration of the homogenization



(c) Humidity of the product



(d) Solvent, UltraTurax



(e) Solvent, sonification

Figure 5.2: Figures (a) to (e) show the results of the tests evaluating the effects of water, time of homogenization, product humidity and type of solvent on the results of the UltraTurax and sonification assisted liquid extraction procedures. (F... fiber, P... paste)

Summary of the evaluation of the suitability of the extraction procedures

UltraTurax assisted liquid extraction
The results did not meet the expectations that fibers have higher contents of allyl isothiocyanate than paste like horseradish products.

With following alterations, still no improvement of the values could be achieved:

- Waive of water
- Variation of the homogenization time
- Use of the drying agent Na_2SO_4
- Variation of the solvent

Ultrasonic assisted liquid extraction
Fibers showed significantly higher values for grated horseradish products (95 % horseradish) than for pastes (65 % horseradish), which is in accordance with the expectations.
With following alterations, minor improvement of the values could be achieved:

- Use of the drying agent Na_2SO_4 led to an adjustment of the values
- Variation of the solvent, proved that dichloromethane is the best solvent with the highest extraction efficiency

According to these results the UltraTurax extraction procedure was discarded and the focus was set on the optimization of the sonification assisted liquid extraction procedure. Besides the more reliable results, the sonification procedure has the additional strength of a much higher sample throughput.

5.1.3 Optimization of the Sonification Assisted Liquid Extraction Procedure

In this chapter the results of the experiments for the optimization of the sonification assisted liquid extraction procedure are represented.

Standard addition

Figure 5.3 shows the results of the standard addition of a grated and a paste like horseradish product. According to the spiking of the samples in equidistant steps with allyl isothiocyanate, one would assume to have a stair like pattern of the analyte. But in this case, a stair like pattern is in the first case only slightly and in the second case not visible at all. Further, the values show very high variations. Also a second isothiocanate which can be found in horseradish, phenetyl isothiocyanate, shows a very strong variation of its values. A standard deviation of 11 % in fibers and of 33 % in pastes was found. In the best case scenario, the values should be constant for each sample type. In comparison, the internal standard, hexyl isothiocyanate, should show constant values under normal conditions over both sample types. Besides the very strong variations of 29 % and 77 %, respectively, of the internal standard, also the values of fibers showed in average higher contents than in the paste like products. Based on these results

it was assumed that there is either a significant problem in the stability of the analytes or the homogeneity of the samples.

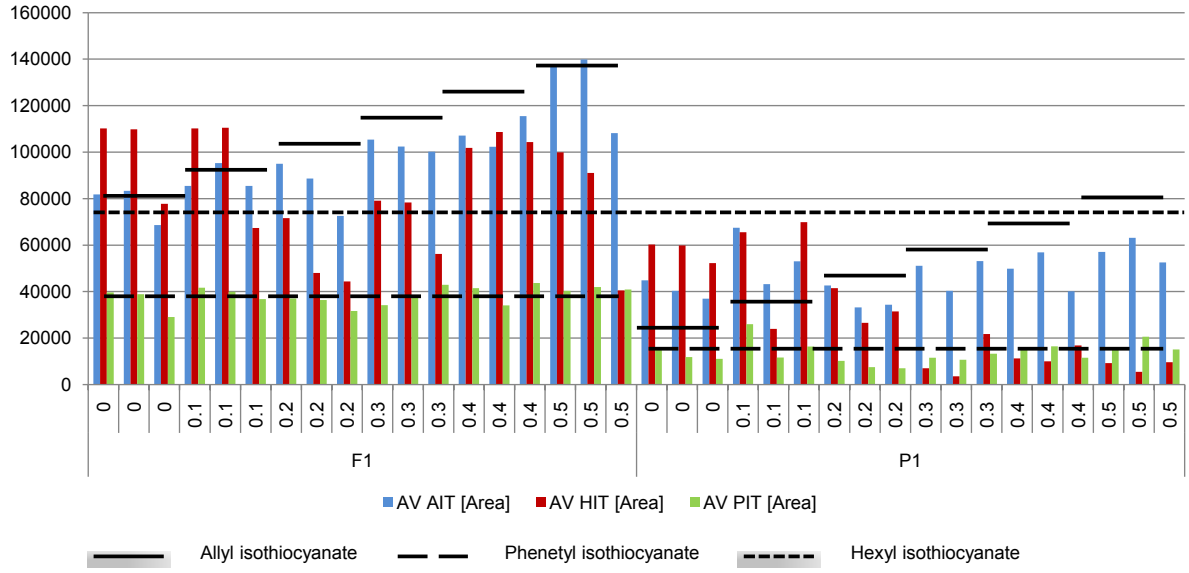


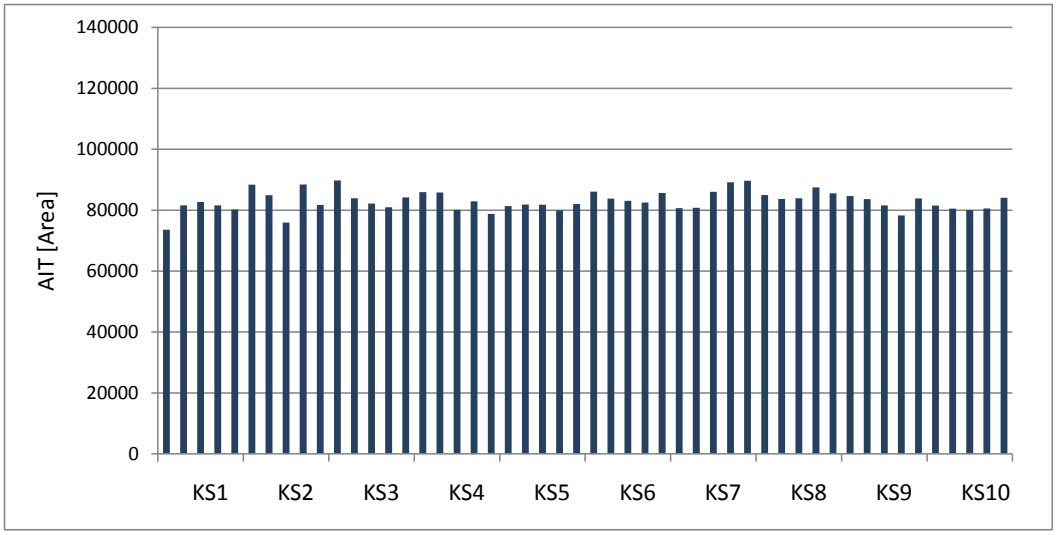
Figure 5.3: The standard addition of a horseradish fiber and a paste product reveals that there is a significant problem in the stability of the analytes or the homogeneity of the samples.

Stability Test

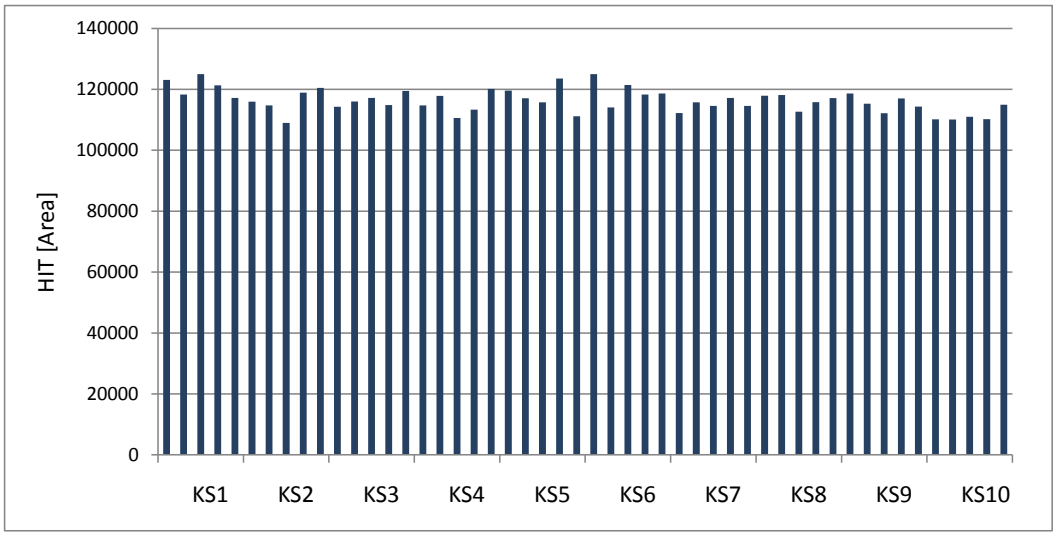
According to the strong fluctuations of the values of the standard deviation, the stability of the analyte and internal standard was tested. In figure 5.4 the representative results of 50 measurements at a injection temperature of 220 °C are represented. The reproducibility of the fivefold injections was calculated to be 3 % for both analyte and internal standard and the reproducibilty of the horseradish simulants was 4 %. This led to the conclusion that the analyte as well as the internal standard are stable at least for the the time of the experiment of 71 hours and in absence of a matrix. Further, the varied injection temperatures had no significant effect on the results.

Displacer

With the proven stability of the analyte and the internal standard, the next aspect to be considered was that the matrix may cause the fluctuations of the values. A possibility to counteract matrix effects is the use of a displacer. The principle function of a displacer is that it blocks active sites due to a higher reactivity compared to the analytes. There are plenty of active sites all over the sample preparation steps and in the analysis, such as glass tubes, liners,



(a) Allyl isothiocyanate



(b) Hexyl isothiocyanate

Figure 5.4: Represented are the results of the five injections of the ten horseradish simulants (K1-10) at an injection temperature of 220°C. Alteration of the injection temperature to 180, 200 and 240 °C had no significant effect on the values.

matrix components etc. In absence of a displacer, active sites can bind the analyte and the internal standard. A displacer now blocks these active sites so that the analyte is not affected. Our tests have shown that triethylamine as displacer leads to a massive improvement of the stability of the values of the internal standard. After the tested concentrations of triethylamine in dichloromethane, the concentration of 0.5 % triethylamine in the extraction solvent showed the best results with a standard deviation of the internal standard of 99 ± 4 %.

Method Check

In order to check the method developed so far, two different brands of grated horseradish products and four different brands of paste like horseradish products were analyzed. For four of the six samples it was possible to obtain reproducible values (see figure 5.5). In the case of sample FA and PF, the values showed high fluctuations, with standard deviations of 58 % and 80 %, respectively.

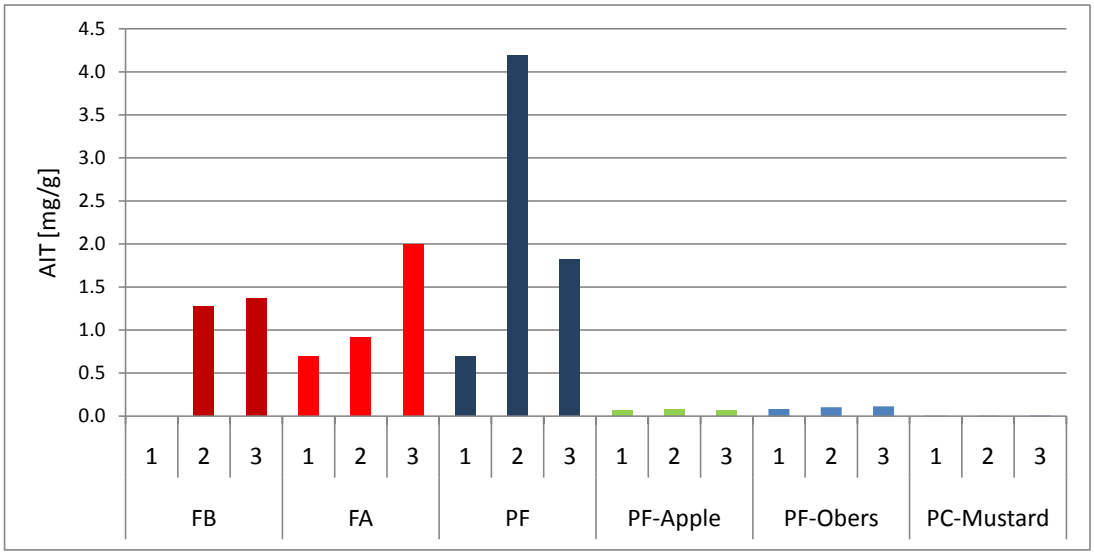
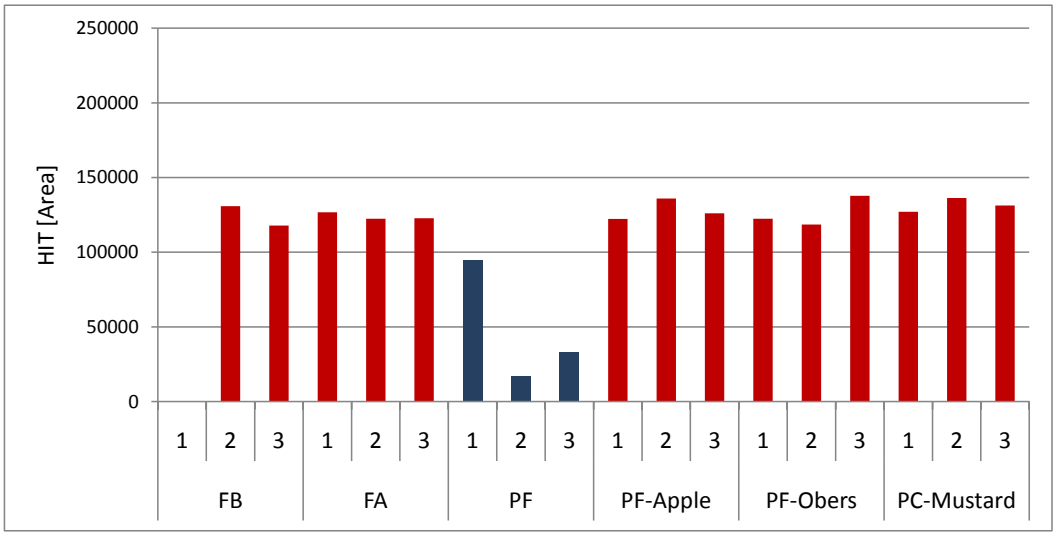
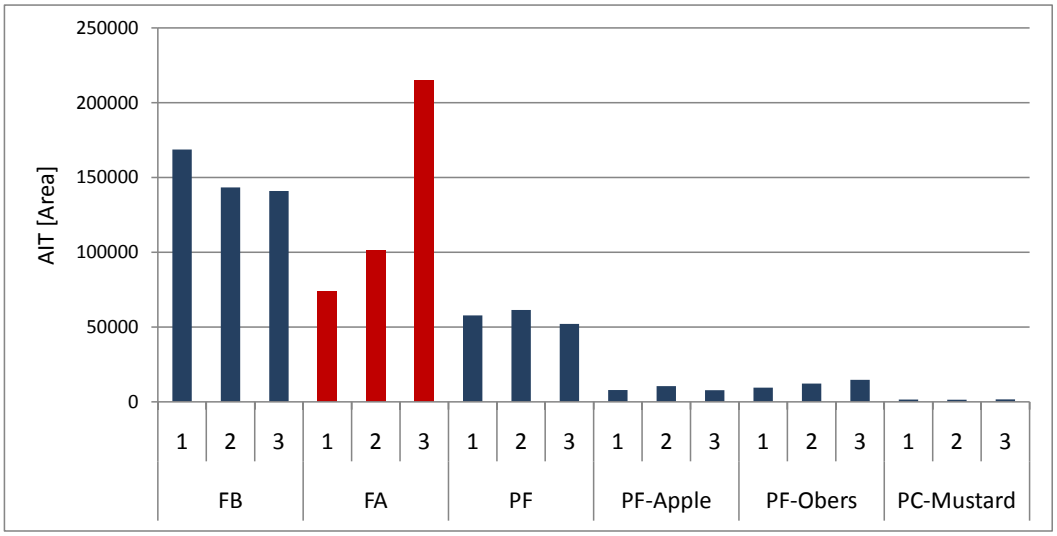


Figure 5.5: The diagram shows the allyl isothiocyanate content in two different brands of grated horseradish products (FB, FA) and four different brands of paste like horseradish products (PF, -Apple. -Obers, PC-Mustard). For a precise description of the samples see chapter 3.4.

A precise look on the data reveals that in the case of sample PF the fluctuations are caused by fluctuations in the values of the internal standard (see figure 5.6a). On the other hand, the fluctuations in the case of sample FA are cause by fluctuations of the analyte (see figure 5.6b).



(a) Hexyl isothiocyanate



(b) Allyl isothiocyanate

Figure 5.6: Represented are the plots of the signal areas of the internal standard hexyl isothiocyanate (5.6a) and the analyte allyl isothiocyanate (5.6b) to show the influence of the fluctuations of the internal standard on the values of sample PF and the influence of the fluctuations of the analyte itself on the values of sample FB.

Determination of the pH of six horseradish products

Paste like horseradish products contain a lot of vinegar as food additive (cf. chapter 3.4). This may cause lower pH values compared to other horseradish products. In order to examine whether a different pH value caused the fluctuations of the allyl isothiocyanate values of sample PF, the pH value was measured for all six samples. As can be seen from the results in figure 5.7, the pH value hardly differed between the samples with pH values of 4.0 ± 0.2. Two samples were repeated with a standard deviation of 0.07 %. Therefore an influence of the pH value seems not to be the explanation for the fluctuation of the values of sample PF.

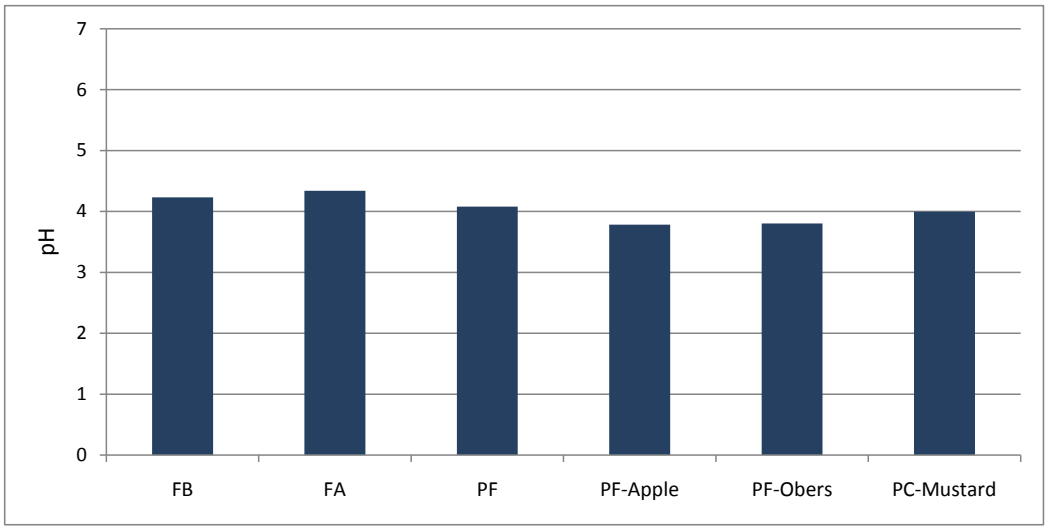


Figure 5.7: Depicted are the results of the pH measurement of two grated and four paste like horseradish products.

Homogeneity of grated horseradish products

Natural products often show a high degree of inhomogeneous distributions of different compounds. To investigate whether sample FA also shows such an inhomogeneity, sample FA was intensively blended with a spatula in a glass beaker and the homogenate was analyzed three times. A comparison of the results to the values of the non homogenized sample FA from the method check (see figure 5.8) reveals that the homogenization led to the determination of highly reproducible values. A standard deviation of 58 % was found for the unhomogenized sample and of 4 % for the homogenzied. These standard deviations indicate that at least grated horseradish products suffer from an inhomogeneous distribution of allyl isothiocyanate within the samples. In an additional test horseradish fibers were chopped in a cryo mill prior to extraction in order to evaluate whether the varying results may be caused by an incomplete cell disruption. However, the results showed no difference compared to the standard sonification assisted liquid extraction.

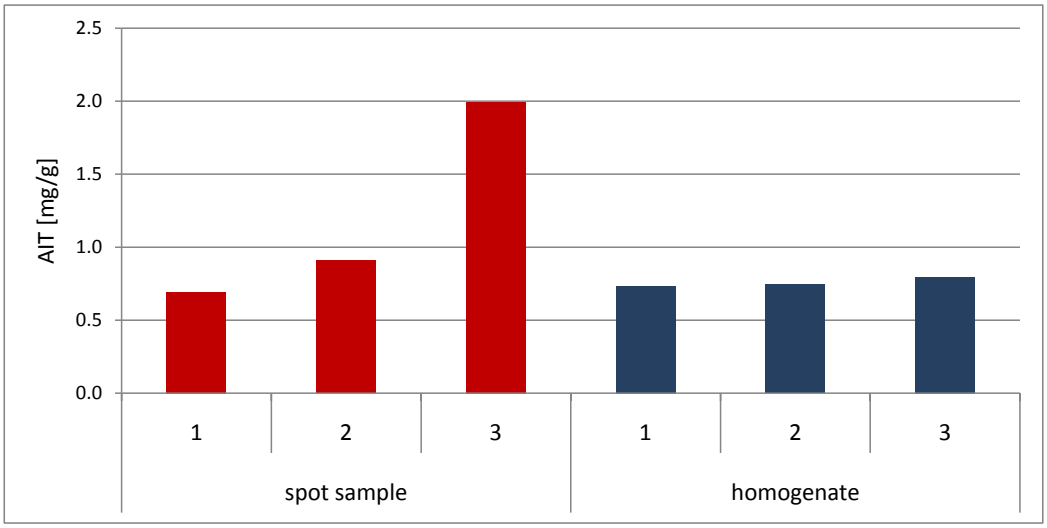


Figure 5.8: Comparison of the content of allyl isothiocyanate before (red) and after (blue) the homogenization of a grated horseradish sample reveals that the distribution of allyl isothiocyanate within fibrous horseradish products reveals great inhomogeneities.

Summary of the Optimization of the Sonification assisted Liquid Extraction Procedure

- The analysis of horseradish simulants over a longer time period revealed that the analyte allyl isothiocyanate and the internal standard hexyl isothiocyanate are stable for at least 71 hours.
- By the use of triethylamine as displacer matrix effects could be minimized, which led to the determination of stable values of the internal standard.
- Determining the pH of six different horseradish products showed that there is no significant difference in the pH between grated or paste like horseradish products. The measured pH values were about 4.0 ± 0.2.
- Homogenization of a grated horseradish product and comparison of the values before and after the homogenization revealed that there is an inhomogenous distribution of allyl isothiocyanate in the product.

5.1.4 Validation of the Allyl Isothiocyanate Method

The validation was performed in conformance with standards (ISO 8466, DIN 32645 [176]). On level 95 % and 99 %, no significant difference was found in the variance test. Also in the linearity test no significant difference was found on level 99%. Further, a relative method standard deviation of 1.5 % was obtained.

Table 5.3: Depicted are the validation results of allyl isothiocyanate.

Status	Validated in conformance with standards
Nr. measurements	18
Nr. replicates	3
Nr. conc. steps	6
Variance test	No significant difference at level 95 % and 99 %
Linearity test	No significant difference at level 99 %
STDP	0.01 [mg/g]
Rel. STDP	1.5 [%]

STD_M stands for method standard deviation

5.1.5 Depth Profiles of Allyl Isothiocyanate in Grated Horseradish Products

The comparison of the depth profiles in figure 5.9 reveals that there are astonishing differences in the homogeneity between the brands. For example, brand A has a much higher inhomogeneity than brand C. Further one can see that also within a brand great differences can occur, as in the case of D1 and D2, for example. In table 5.4 the corresponding standard deviations and average values are listed. In summary the standard deviation of the values within the depth profiles varies from 11 to up to 63 %. So there are enormous differences in the homogeneity. Further one can see that great differenences occur not only between brands. There are also significant differences within the brands, as is the case of brand A or D. For the homogenates the standard deviation was between 3 to 10 %. Therefore it could be proven that intense mixing allows the formation of reproducible values. A comparison of the means of the depth profiles with the corresponding means of the homogenates showed a congruence of 53 to 92 %. It is assumed that within the homogenization process, a loss of the analyte occurs. In the depth profiles the avarage content of allyl isothiocyanate differs between 1.76 mg/g fresh weight to 0.59 mg/g fresh weight. For the homogenates the values differ from 0.44 to 1.36 mg/g fresh weight.

Measurements of fibrous horseradish products (see chapter 5.4.3) revealed that processing of horseradish roots to grated products leads to a loss of sinigrin with residual amounts close to zero. Therefore the pungency of these products cannot instantaneously be formed by hydrolysis

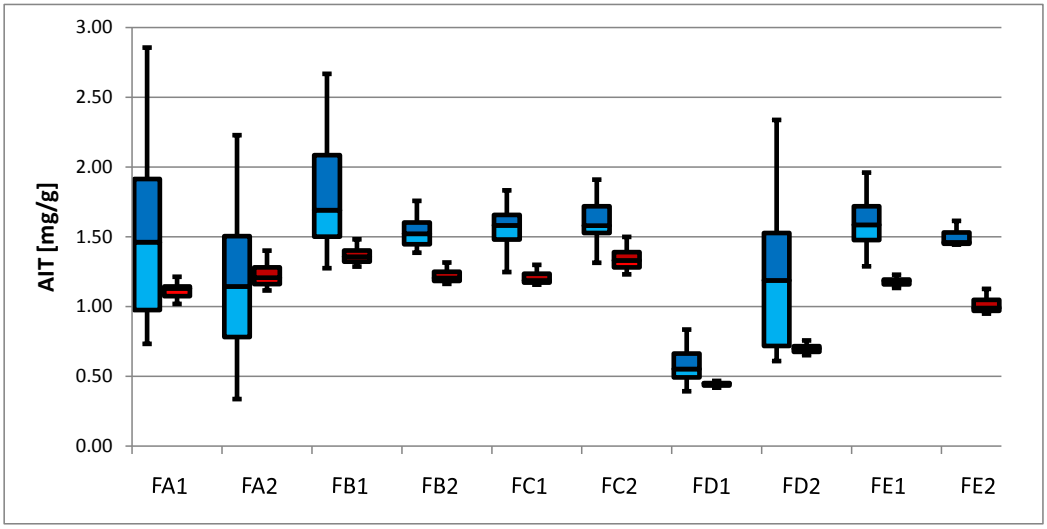


Figure 5.9: BoxPlot of the depth profiles of five different brands FA to FE (cf. chapter 3.4). The blue bars describe the distribution of the values of the depth profiles whereas the red bars describe the values of the corresponding homogenates.

of sinigrin in presence of the endogenous *myrosinase*, when the spatial separation of these substances is eliminated by chewing. So somehow the degradation product of sinigrin, allyl isothiocyanate, needs to be trapped during the processing of horseradish roots. According to their carbon hydrate residue isothiocyanates are fat-soluble. A closer look on the ingredients of the analyzed grated horseradish products (see chapter 3.4) shows that all of them contain around 5 % of rape seed oil or sunflower oil. Therefore allyl isothiocyanate is trapped in these oils during processing and is released in some extend at consumption. The intensive pungent and lachrymatic experience while eating is caused by an increased evaporation of allyl isothiocyanate at body temperature. Inhomogeneous distributions of the analytes must therefore correlate with the distribution of the oil in the products. For example, in case of the sample FA great differences in the distribution of oil could be observed during sample preparation. At the top of the sample the fibers were a bit dry and at the bottom a lot of oil was found. This was also reflected in the results of the depth profiles as from the top to the bottom the content of allyl isothiocyanate increased. This was not the case for other products with a smaller distribution of the content of the analyte. Further this theory can explain why intensive blending of the fibers leads to reproducable values. While blending the fibers, the oil in the products is redistributed over the fibers and as the fiber thickness is very constant the surface of the fibers is proportional to the weight and therefore to the amount of oil.

Table 5.4: Key parameters of depth profiles of grated horseradish products of 5 different brands

Samples	STD. within DP samples [%]	STD. within H sample [%]	AV depth profile [mg/g]	AV homogenized [mg/g]	Comparison AV H/AV DP [%]
FA1	47	7	1.57	1.10	70
FA2	63	10	1.33	1.23	92
FB1	20	6	1.76	1.36	77
FB2	20	6	1.56	1.22	78
FC1	16	6	1.52	1.21	79
FC2	19	8	1.62	1.34	82
FD1	25	4	0.59	0.44	75
FD2	57	6	1.31	0.69	53
FE1	24	3	1.71	1.17	69
FE2	11	8	1.48	1.02	69

5.1.6 Evaluation of the Decrease in Pungency of Two Different Brands

With a slope of - 0.0239 for brand FB and a slope of - 0.0156 for brand FA the desired difference in the decrease of pungency between the two brands could not be confirmed. As the trend lines in figure 5.10 show, their is no significant difference in the decrease between these two brands. However the product of brand FB has about 0.7 mg/g fresh weight more allyl isothiocyanate as the competitor, which correlates with a more pungent product of brand FB.

As previously discussed, sinigrin is nearly completely hydrolyzed when horseradish roots are processed to fibrous products (see chapter 5.4.3). Therefore the measured allyl isothiocyanates were all dissolved in the rape seed oil. And as further there is no difference in the ingredients of the products from brand FB to FA, there is no reason why the decrease of the analyte should be greater in either one of these products compared to the other. However, as the results show, the products of FB always had a higher content of allyl isothiocyanate than the products of FA. This can either be explained by the use of horseradish roots with a higher content of sinigrin in case of brand FB or by an optimized process of FB, where somehow the trapping of allyl isothiocyanate in the oil is more efficient and the loss of the isothiocyanates during processing is reduced.

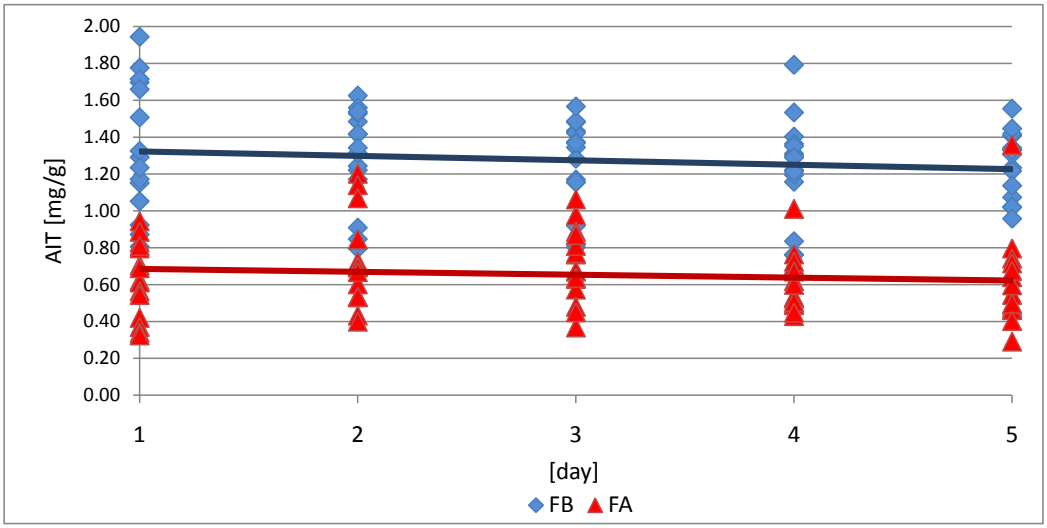


Figure 5.10: Depicted are the results of the evaluation of the decrease in pungency of two different brands. In blue the results of brand FB and in red the results of brand FA are highlighted. According to the trend lines in red and blue, there is no significant difference in the loss of pungency between these two brands.

5.2 Method Development: Enzyme Activity Determined by UV/VIS Spectroscopy

For the first test the isolation of *myrosinase* from grated horseradish products was done at room temperature. However no decline in optical density could be observed for extracts from this isolation procedure. As the centrifuge went very hot, it was assumed that maybe the enzyme was inactivated. Based on this assumption, the second test was carried out at approximately 4 °C. Again no enzyme activity could be observed. As there is no information about the processing of grated horseradish products, it can not be excluded that the enzyme some how gets inactivated during the process. In order to check this also roots were analyzed, but there was still no decline in optical density.

In comparison to the procedure for the determination of the enzyme activity published by Li *et al.* [174], others also added ascorbate to the reaction mixture [20, 97]. Therefore 130 µL of a 0.25 mM solution of ascorbate were added to the reaction mixture of the second trial and the determination of the enzyme activity of the extracts isolated at 4 °C repeated. This was done for extracts from roots as well as for grated horseradish products. Interestingly the extracts from horseradish roots showed an enzyme activity in presence of ascorbate, but likewise extracts from grated horseradish products still didn't shown any activity at all.

Research published by Shikita *et al.* [97] has shown that sulfate is the only degradation product of glucosinolate hydrolysis that inhibits the activity of *myrosinase*. As desulfoglucosinolates neither act as substrates nor as inhibitors, the sulfate moiety at glucosinolates appears to be required for the binding to the enzyme [177]. Therefore the inhibiting effect of sulfate is related the blockade of the binding site. An explanation for the absence of the enzyme activity in grated horseradish products could be the food additive sodium metabisulfite (E224), which is found in all horseradish products for conservation purposes (see chapter 3.4). It is possible that metabislufite and its hydrolysis product hydrogen sulfite inhibit *myrosinase* activity in the same way as sulfate. The inhibiting effect of sodium sulfite on *myrosinase* was allready proven by Li *et al.* [178]. In order to evaluate the effect of sodium metabisulfite, further experiments have to be done.

The effect of ascorbate as a co-factor of *myrosinase* was discussed in chapter 2.5.3. It is interesting that *myrosinases* from different *Brassicaceae* plants show different degrees of activation in presence of ascorbate. Wilkinson *et al.* [179] investigated the effect of the ascorbate concentration on seven different *Brassicaceaes* (see table 5.5). His group found out that ascorbate independent *myrosinase* activity extremely depends on the species. For some plants ascorbate is crucial for their *myrosinase* activity, e.g. for red cabbage and turnip, which reach only 1 and 3 % respectively of their maximal enzyme activity in absance of ascorbate. In comparison, white mustard and radish remain 72 and 30 % of their maximal enzyme activity, respectively, in absence of ascorbate. The results of our measurements lead to the assumption that the *myrosinase* of horseradish belongs to the family of *myrosinases* that have an almost absolute requirement for ascorbate. However, these results are in conflict with the result of Li *et al.* [8], who did not add ascorbate to the reaction mixture. For different horseradish genuses he found *myrosinase* activities between 1.2 - 57.1 units/g of dry weight.

Table 5.5: Represented are the results of the ascorbate independent *myrosinase* activity and the concentration ranges of ascorbate leading to *myrosinase* activities higher then 90 % published by Wiklinson *et al.* [179].

Species	Ascorbate independent *myrosinase* activity [% maximal]	activity > 90 % [mM ascorbate]
White mustard	72	0.4 - 7.0
Radish	30	0.8 - 2.0
Brussels sprouts	10	0.65 - 1.5
Cauliflower	4	0.65 - 5.
Red cabbage	1	0.9 - 3.5
Turnip	3	0.75 - 0.99
Watercress	5	1.0 - 7.0

5.2.1 Dependence of the Enzyme Activity on the Ascorbate Concentration

The investigation of the influence of the ascorbate concentration on the enzyme activity revealed an increase of the activity at low ascorbate concentrations and a decrease in activity at high levels of ascorbate (see figure 5.11). The maximum of enzyme activity is reached at an ascorbate concentration of 0.75 mM. This activating and inhibiting nature of ascorbate is a common phenomenon to the different *myrosinases* from *Brassicaceae* plants [179] and is related to a binding competition between substrate and ascorbate at high concentrations of ascorbate [97].

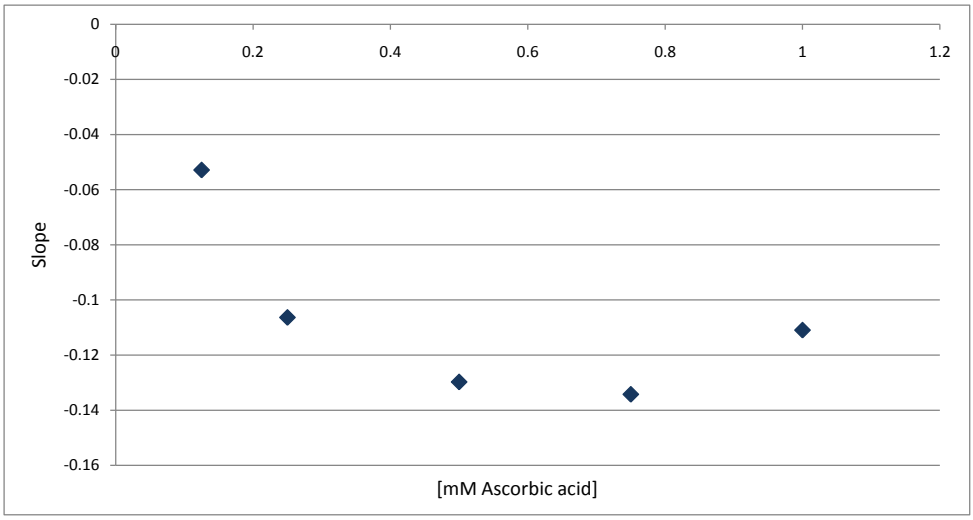


Figure 5.11: The slope of the optical decline is proportional to enzyme activity and increases with the addition of ascorbate, reaches a maximum at 0.75 mM ascorbate and decreases with higher levels, due to a binding competition of sinigrin and ascrobate.

If a curve is plotted over the data points in figur 5.11, an approximation to a concentration of 0 mM ascorbate shows that in absance of ascorbate, none or just a small proportion of the maximal enzyme activity remains. As discussed above, *myrosinases* from different *Brassicaceae* plants have different dependences on the presence of ascorbate in order to show an enzyme activity. It was also mentioned that this data is in conflict with data ofm Li *et al.* [8], who analysed the enzyme activity of 22 horseradish genuses without the addition of ascorbate to the reaction mixture. A possible explanation for our results as well as for the results of Li *et al.*, who showed a great difference in the enzyme activity of different genuses, is the different response to ascorbate concentration of *myrosinase* isoenzymes [180]. Hence, the set of isoenzymes determines whether their is an activity in absence of ascorbate and how high this activity is under certain parameters.

Table 5.6: This table shows the results of the enzyme activities at given ascorbate concentrations. The enzyme activity in units per g dry weight was calculated from the activity in units per g fresh weight by the assumption that a horseradish roots contains 85 % of water (cf. chapter 2.1)

Ascorbic acid [mM]	[units/cm^3]	Total Units	Units per g FW	Units per g DW
0.125	0.23	7.0	2.3	15.6
0.25	0.47	14.1	4.7	31.5
0.5	0.57	17.2	5.7	38.4
0.75	0.59	17.8	5.9	39.7
1	0.49	14.7	4.9	32.8

In order to check the plausibility of the data and to evaluate the developed method, the results were compared with the results of Bellostas *et al.* [181] and Li *et al.* [8]. Bellostas *et al.* performed the direct spectrophotometric enzymatic assay in presence of 0.25 mM ascorbate. He found enzyme activities for *B. carinata*, *B. Oleracea* and *B. napus* between 0.38 - 0.96 U/cm^3, which is in the same magnitude as the results of our measurement (cf. table 5.6). Li *et al.* determined the enzyme activity of *myrosinase* from horseradish roots and found activities from 1.2 to 57.1 U/g of dry weight. Again comparison with our data revealed a good accordance, however we have to consider that the results of Li *et al.* were obtained in absence of ascorbate.

5.3 Method development LC-MS/MS Citric and Isocitric Acid

The evaluation of the results from the measurement of different concentrations of analyte mixtures revealed that the product ions with a m/z of 111.00 and 84.90 from citric acid and 110.95 and 173.05 from isocitric acid are each affected by the other analyte. Therefore the m/z 87.05 was selected for citric acid and the m/z 73.00 for isocitric acid as quantifier ions, although the highest intensity was found for both analytes for the m/z of 111.00 and 110.95, respectively. In the case of tricarballylic acid the product ions were not affected by the other analytes due to the different precursor m/z. For tricarballylic acid the most intensive m/z of 157.00 was chosen as quantifier ion.
Retention of polar substances on a C18 column is achieved with ion pairing. For that purpose the mobile phases methanol and water were acidified with formic acid. A comparison of 0.1 to 0.3 % of formic acid in the mobile phases revealed better retention for the higher concentration of formic acid. This is in accordance with the consideration that the level of acidity correlates with the protonation of the carboxylic acid functions of the analytes. The higher the degree of protonation, the stronger is the interaction with the stationary phase due to the lack of the negative charge. According to that the dilution of the analytes in methanol-water with 0.3 % formic acid was tested. This led to higher intensities, however this positive effect was compensated by an unsymmetrical peak shape. So MeOH:H_2O=1:1 without formic acid was

used for extraction and dilution of samples. Tests of isocratic and programmed mobile phase conditions revealed that the sensitivity increased with an increase of the proportion of water in the mobile phase. All tests came to the result that the best separation of analytes is achieved under isocratic conditions with 90 % of 0.3 % formic acid in water and 10 % of 0.3 % formic acid in methanol in the mobile phase. But due to the similar structure of citric and isocitric acid, these conditions only increased the separation between these two analytes and the internal standard tricarballylic acid. Furthermore, an overlap of the analytes still occured (see figure 5.12). This is the justification and explanation why the use of LC-MS/MS is needed for the determination of citric and isocitric acid.
The optimization of the ESI parameters showed an increase of intensity when the interface voltage was altered to - 1 kV and the resolution of the first quadrupole was set to unit and that of the third quadrupole to low.

A summary of the fully developed method with its optimized parameters is given in chapter 3.1.4.

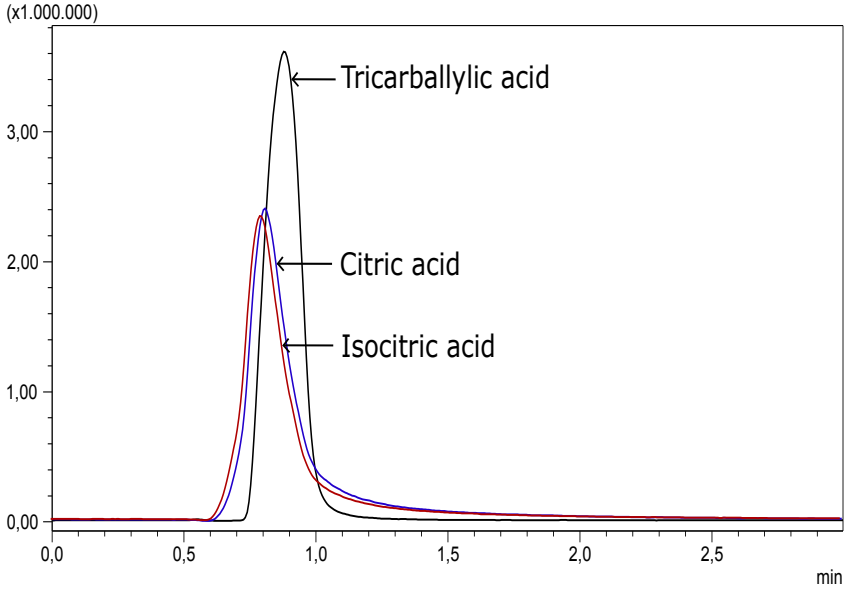


Figure 5.12: Shown is the chromatogram (TIC) of a solution of 3 mg/L of citric and isocitric acid and 2 mg/L of tricarballylic acid.

5.3.1 Calibration of Citric and Isocitric acid

The calibration of 0.1 - 10 mg/L of citric and isocitric acid showed a loss of signal intensity for all MRM events of the internal standard tricarballylic acid with 2 mg/L for the calibration points 5 and 10 mg/L. An explanation for this effect could be the occurrence of an ion suppression. Generally the term ion suppression describes the influence of a component on the ionization

of a coeluted analyte. The origin and mechanism of this effect is multifaceted and strongly depends on the ionization technique. High concentrations, basicity, mass and the elution at the same retention time are factors which make a compound a possible ion suppressor [182]. In the case of electrospray ionization, two theories try to explain the presence of ion suppression. The first theory relates signal suppression to a reduced solvent evaporation, triggered by an increase of the surface tension and viscosity of droplets in presence of high amounts of interfering compounds and therefore a reduced transition of analytes in to the gas phase [183, 184]. The second theory describes how ion suppression occurs due to a limited supply of excess charge in droplets. Which compound will out-compete the others is related to its basicity. Another effect based on saturation is when an analyte occupies the surface and therefore inhibits the ejection of ions inside the droplet. In this case the suppressed ion is the one with the lower surface activity [185]. In order to get the biggest calibration range, the calibration points 1, 2, 3, 4 and 5 mg/L were examined for the occurrence of the ion suppression. As shown in figure 5.13, ion suppression starts at the calibration point of 4 mg/L.

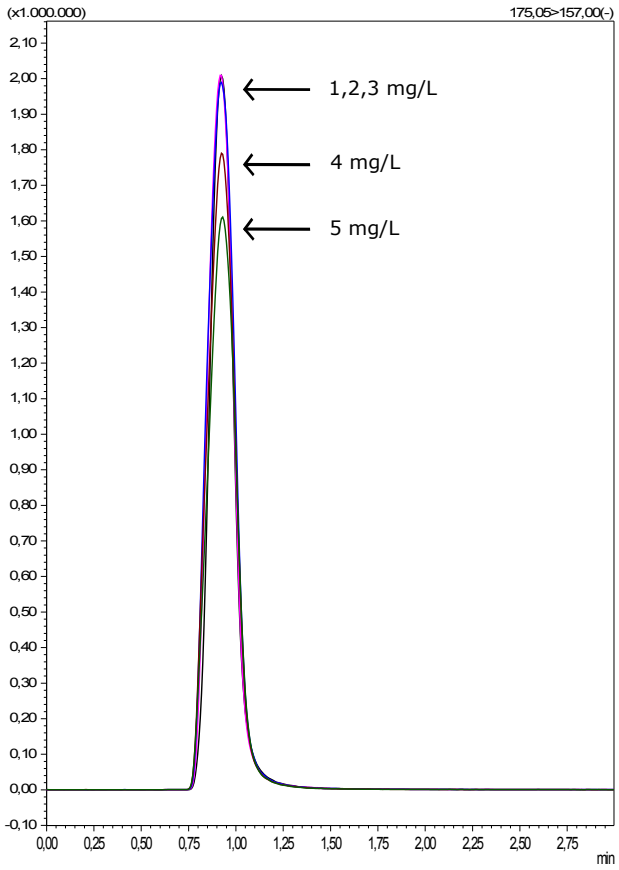


Figure 5.13: The chromatogram shows the MRM transition 175.05 > 157.00 of tricarballylic acid of the calibration points 1,2,3,4 and 5 mg/L. As can be seen, the ion suppression of tricarballylic acid starts in presence of 4 mg/L of citric and isocitric acid.

As a consequence of the ion suppression, the calibration range was shortened from 0.1 to 3 mg/L and the calibration repeated, whereby each calibration point was prepared in three replicates. Interestingly the calibration points of citric as well as of isocitric acid were better fitted with a second degree polinomial (cf. figure 5.14 and table 5.7). Maybe this polynomial behavior can be explained by an additional increase of the signal with increasing concentrations of citric and isocitric acid, as the increasing concentration strengthens citric and isocitric acid in the competition for charge against tricarballylic acid (2 mg/L). Still, ion suppression has a rather great influence on the values. This is shown by the comparison of the standard deviation within the three replicates, which is in average 0.3 % for tricarballylic acid, 2.3 % for citric acid and 1.5 % for isocitric acid, with the standard deviation of tricarballylic acid calculated over all values, which has a standard deviation of 11.8 %. In section 5.3.3, the results from the linear and polynomial calibration curve will be compared.

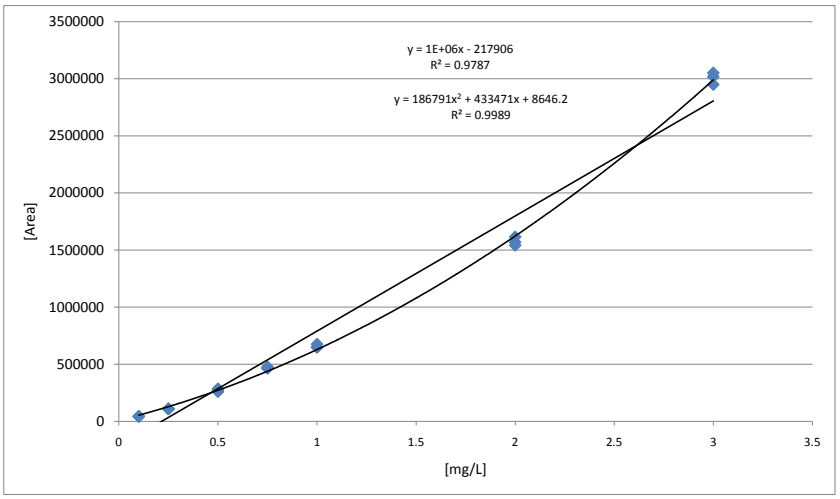


Figure 5.14: With an coefficient of determination of 0.9989 compared to 0.9787 of the linear fit, the polynomial fit shows a better approximation of the citric acid calibration points. This is also the case for the calibration of ioscitric acid.

Table 5.7: Key parameters of the citric and isocitric acid calibration

	Citric acid		Isocitric acid	
	Linear	polynomial (2°)	Linear	polynomial (2°)
Slope	1007977	186791	1066916	173821
"Slope" 2	-	433471	-	532301
Intercept	-217906	8646	-160266	50555
R2	0.9787	0.9989	0.9817	0.9973
LOD® [mg/L]	0.17	-	0.16	-
LOQ® [mg/L]	0.60	-	0.55	-

® LOD and LOQ were calculated based on the calibration method.

5.3.2 Validation of the Citric and Isocitric Acid Method

It was possible to perform a validation in conformance with standards (ISO 8466, DIN 32645 [176]). Variance as well as the linearity test were positive. According to the problem with ion suppression, the method's standard deviation is rather bad and requires improvement.

Table 5.8: Depicted are the validation results of citric acid.

Status	Validated in conformance with standards
Nr. measurements	18
Nr. replicates	3
Nr. conc. steps	6
Variance test	No significant difference at level 95 % and 99 %
Linearity test	No significant difference at level 99 %
STD_M	1.0 [mg/g]
Rel. STD_M	20 [%]

STD_M stands for method standard deviation

5.3.3 Amount and Distribution of Citric Acid in Horseradish Roots

The sample preparation procedure produced very good results, with a standard deviation of the internal standard of 6 %, a standard deviation of a threefold injection for citric acid of 2.5 % and a standard deviation for the preparation of three replicates (sample RB1 1i) for citric acid of 2.3 % and for isocitric acid of 6.9 %.
The results of the determination of the amount and distribution of citric and isocitric acid in two different roots (see table 5.9) revealed average values (both roots) for citric acid of 6.9 mg/g for the outer part and 11.4 mg/g for the inner part. In case of isocitric acid, the average was 0.35 mg/g for the outer part and 0.44 mg/g for the inner part. As the results show, the amount of citric acid is significantly higher in the core of the root compared to the outer part. In average the concentration of the outer part is approximately 61 % of the values found in the

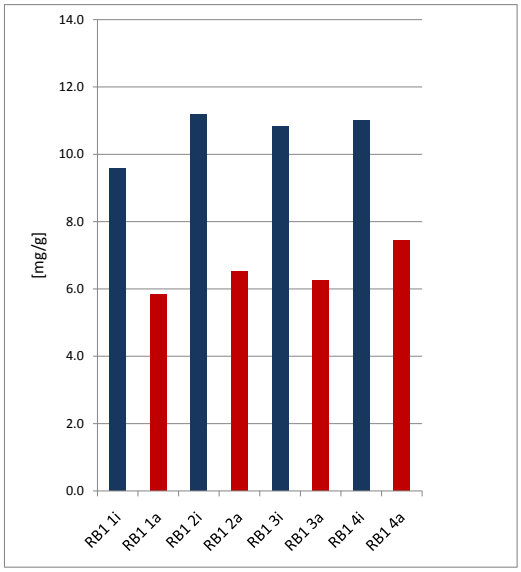
core. This is very interesting as citric acid was found to inhibit myrosinase activity [178]. If we think about the defense mechanism of glucosinolates, which occurs after damage to the outer cells in presence of pests, it makes sense to have a reduced concentration of citric acid in the shell of the root, as high enzyme activities favor a successful defense. A comparison of the two analyzed roots (see figure 5.15) shows that the citric acid concentrations within the samples of the core and of the outer part respectively are with a standard deviation of 8.5 % relatively homogeneous. Whereby root RB2 contains in average 1.1 mg/g more citric acid than root RB1. A closer look at the results of isocitric acid shows a similar behavior as citric acid in the case of root RB1, just the results of root RB2 differ from the pattern found for the others. As far as a literature search showed, there is only the work of Pohloudek-Fabini [186] (1955) in which the citric acid concentration in horseradish was determined as part of an investigation on the citric acid content in a range of vegetables. For horseradish he found 675 mg citric aicd per 100 g fresh weight. However, the data were obtained by just measuring one sample per vegetable species and therefore ignoring the dependence of the organic acid concentrations on the type of species, growth and harvesting conditions.

A comparison of the values calculated by the linear and polynomial calibration curve (see table 5.9) reveals that the values calculated by the polynomial curve are approximately 6.5 % higher. According to the polynomial shape the difference between the values is higher for lower signals. However, due to the relative method standard deviation of 20 % and a coefficient of determination of the linear calibration curve of 0.9789, the influence of the polynomial shape plays only a minor role.

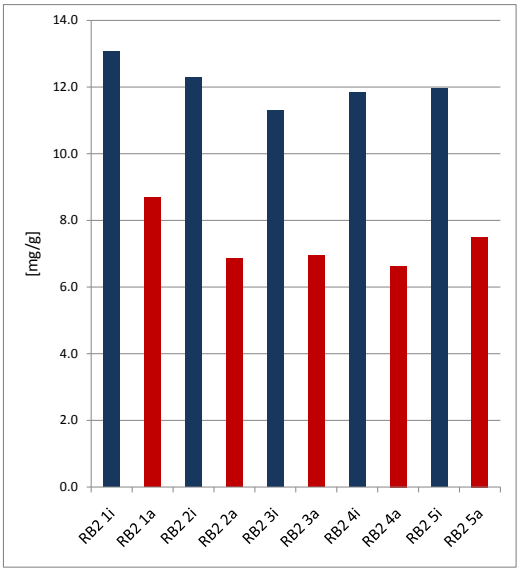
Table 5.9: Summary of the results of the citric and isocitric acid profile of horseradish roots.

	Citric acid				Isocitric acid	
Root	RB1		RB2		RB1	RB2
Calibration	Linear	Polynomial	Linear	Polynomial	Linear	Linear
AV a [mg/g]	6.5	7.3	7.3	8.2	0.3	0.4
AV i [mg/g]	10.6	11.4	12.1	12.5	0.5	0.4
AV a / AV i [%]	61.2	64.4	60.5	65.8	72.1	91.9

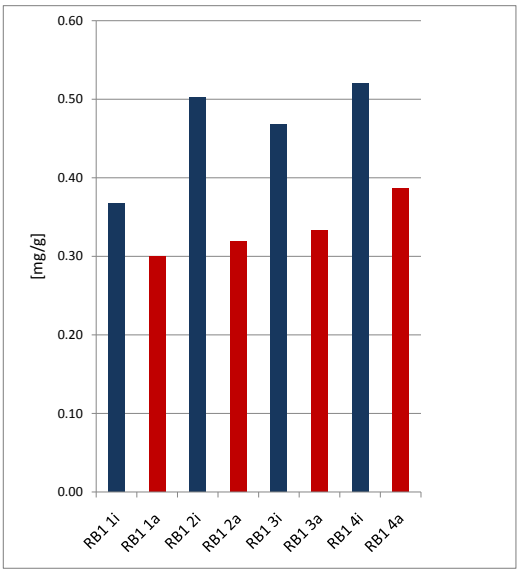
[AVi] Average value of the root samples of the inner part.
[AVa] Average value of the root samples of the outer part.



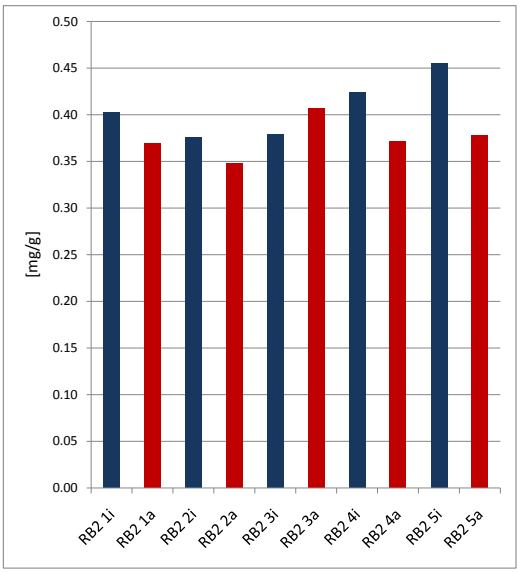
(a) RB1 citric acid



(b) RB2 citric acid



(c) RB1 isocitric acid



(d) RB2 isocitric acid

Figure 5.15: Depicted are the results of the determination of the amount and distribution of citric and isocitric acid in horseradish roots. Red bars indicate the results of the outer part of horseradish roots and blue bars the results of the core. Interesting is the higher concentration of the acids in the core. This is in coincidence with the theory of the pest defense, as citric acid would reduce the *myrosinase* activity and therefore the evaporation of the defense break down products.

5.4 Method development LC-MS/MS Glucosinolates

The generation and optimization of the MRM events was straight forward. The selected transition for the quantifier ion was 358.10 > 96.95 and for the qualifier ions 358.10 > 96.00 and 358.10 >75.00.
With a two-step gradient and a flux of 0.2 mL/min. (0 % B - 20 % in 4 min. - 100 % B in 3 min. - 100 % B for 1 min. - 0 % B held 1 min.) a double peak was generated. With the increase of the flux to 0.4 mL/min., the front peak was transferred to a shoulder of the back peak. In order to evaluate whether the doubled peak shape is a consequence of a high concentration of sinigrin, the injected concentration was altered to 1 mg/L. However, this did not lead to an improvement of the peak shape. The gradient was changed to a simple gradient starting from 0 to 100 % of mobile phase B in 5.5 minutes, which was held for 1 minute. and then the column was re-calibrated with a concentration of 0 % of B for 1.5 minutes. The peak shape was investigated for the starting concentrations of 5, 10, 15, 20, 25 and 50 % mobile phase B. First, an increase of the intensity of the peak accompanied with the formation of a more and more symmetrical peak was found. At 25 % of mobile phase B the most symmetrical peak was gained, when the proportion of B was further increased, the intensity decreased and a tailing could be observed (cf. figure 5.16). Further investigations revealed that a gradient is not necessary and an isocratic method with 25 % of mobile phase B was applied for further measurements.
The optimization of the ESI parameters showed an increase of intensity when the interface voltage was changed to - 1 kV, the heating gas flow was set to 15 L/min., the drying gas flow altered to 5 L/min. and the resolution of the first quadrupole set to low and the third quadrupole to unit.

5.4.1 Calibration of the Sinigrin

The calibration of sinigrin showed a nice linear behavior over two decimal powers with a coefficient of determination of 0.9997. The prepared three replicates per calibration point had in average a standard deviation of 3 %. A calculation of the LOD and LOQ, based on the calibration method, revealed values of 0.07 mg/L and 0.26 mg/L respectively.

Table 5.10: Key parameters of the sinigrin calibration

	Slope	Intercept	R2	LOD® [mg/L]	LOQ® [mg/L]
Sinigrin	429247	-9114	0.9997	0.07	0.26

® LOD and LOQ were calculated based on the calibration method.

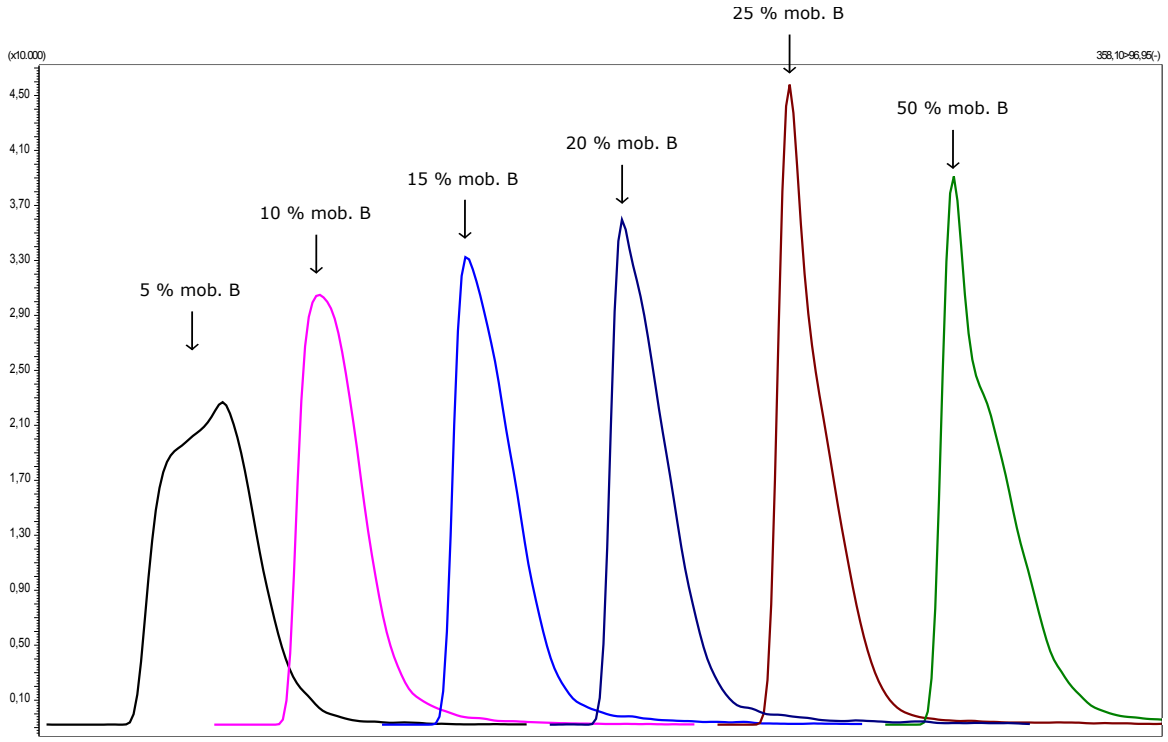


Figure 5.16: Represented is the dependence of the peak shape of sinigrin on the amount of mobile phase B. The increase of mobile phase B is accompanied with an increase of the intensity and nicer peak shapes. When the concentration of mobile phase B is further increased, a tailing occurs.

5.4.2 Validation of the Sinigrin Method

The validation could be performed in conformance with standards (ISO 8466, DIN 32645 [176]). The variance test found no significant differences on level 95 and 99 (%) and the linearity test was also positive. Again, no significant differences was found on level 99 (%). Additionally a relative method standard deviation of 2 % was obtained.

Table 5.11: Depicted are the validation results of sinigrin.

Status	Validated in conformance with standards
Nr. measurements	18
Nr. replicates	3
Nr. conc. steps	6
Variance test	No significant difference at level 95% and 99%
Linearity test	No significant difference at level 99%
STD_M	0.1 [mg/g]
Rel. STD_M	2 [%]

STD_M stands for method standard deviation

5.4.3 Amount and Distribution of Sinigrin in Roots and Processed Horseradish

The preliminary tests of the sample preparation showed that *myrosinase* is sufficiently inactivated after 15 minutes at 75 °C. Further extracts of horseradish roots had to be diluted 1 + 99 in order to obtain values within the calibration curve. In the case of grated horseradish products the extracts had to be diluted by 1 + 9 and an injection volume of 5 µL was needed in order to use the calibration curve. The standard deviation of a threefold injection was 1.2 %. With these results a final method was generated (see chapter 3.1.4) and was applied to generate sinigrin profiles of horseradish products.

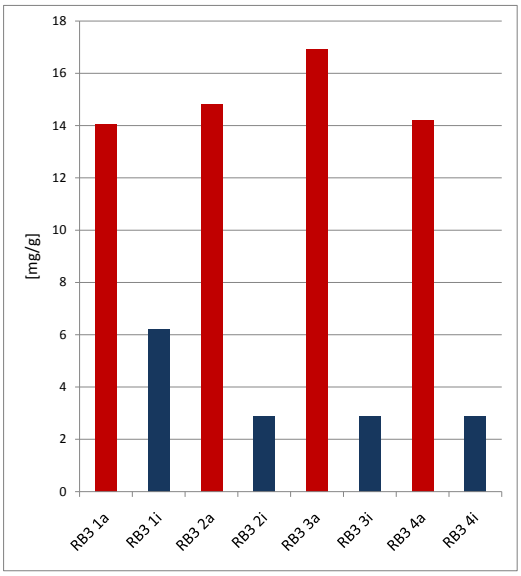
The determination of the amount and distribution of sinigrin in two roots and two different brands (see table 5.12) of grated horseradish products revealed average values of 3.7 and 4.2 mg/g fresh weight for the core of the analyzed horseradish roots respectively. In the outer part average values of 15.0 and 14.5 mg/g fresh weight were found. The standard deviations of the values of the core are 45 or 30 % and of the outer part 9 and 16 % respectively. A comparison of the roots (see figure 5.17) shows a higher amount of sinigrin in the core segment one (head of the root) in both cases, which is mainly responsible for the high standard deviations of the core values. The tendency of a decrease of the amount of sinigrin from the head to the tip of the root in the outer layer can only be assumed for root RB4. More roots need to be analyzed in order to consolidate these tendencies, according to the nature of strong variations of the values of natural products. In contrast, a comparison of the core values to the outer layers reveal significantly higher values for the outer layers, which are approximately 400 % higher than the core values. These results are in accordance with the theory that glucosinolates are involved in pest defense. First, plant pests destroy cells from the shell, which is accompanied by the removal of the spatial separation of glucosinolates and *myrosinase*, releasing the corresponding defense active isothiocyanates by hydrolysis. Therefore higher amounts of sinigrin in the shell correlate with a strong defense capability. The results of the grated horseradish products (see figure 5.17) show very low values, which are smaller than 0.007 mg/g fresh weight. An explanation could

be a strong hydrolysis of glucosinolates, as a consequence of cell injuring during processing. For example, cutting of brussels sprouts, cauliflower or cabbage into 5 mm cubes results in a loss of the total glucosinolate content to less then 75 % of the initial amount within the first 6 hours [96] (cf. chapter 2.6.9). With additional time the amount of glucosinolates decreases further and approaches zero.

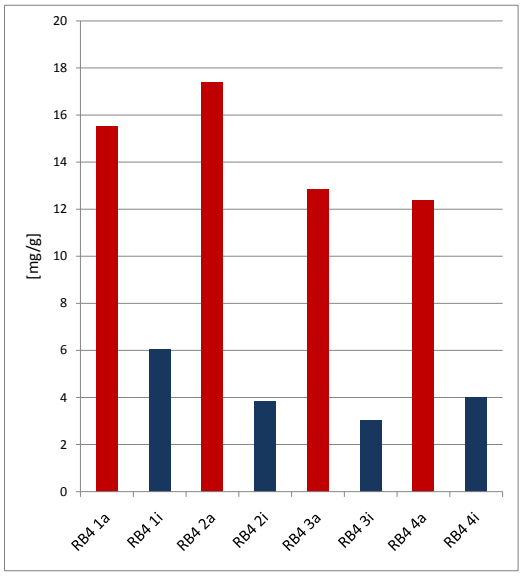
Table 5.12: Summary of the results of the sinigrin profiles of roots and grated horseradish products.

	RB3	RB4	FA	FB
AV i [mg/g]	3.7	4.2	0.007	0.005
AV a [mg/g]	15.0	14.5	-	-
SD i [%]	45	30	5	8
SD a [%]	9	16	-	-

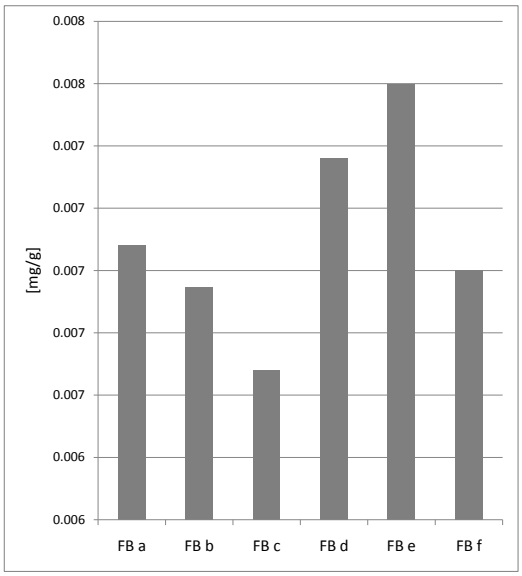
[AV] stands for average value
[SD] stands for standard deviation
[i] stands for core samples
[a] stands for samples of the outer part



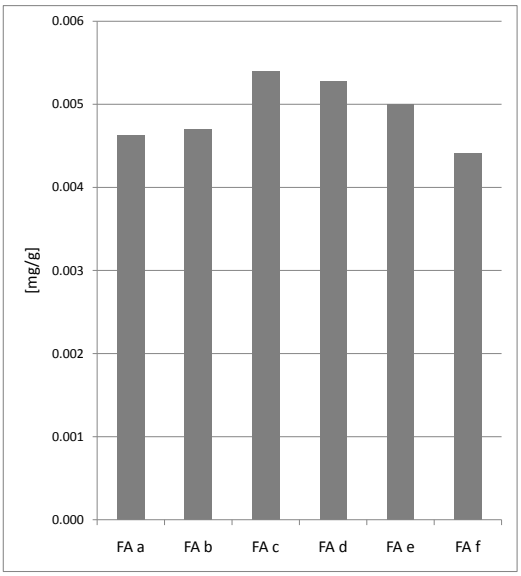
(a) root RB3



(b) root RB4



(c) fiber FB



(d) fiber FA

Figure 5.17: Depicted are the results of the determination of the amount and distribution of sinigrin in horseradish roots and grated horseradish products. Red bars indicate the results of the outer part of horseradish roots and blue bars the results of the core. Interesting is the higher concentration of sinigrin in the outer part of horseradish roots (RB3, RB4). This is in coincidence with the theory of the pest defense, as pests would first destroy cells of the outer shell and therefore higher concentrations of sinigrin in the outer part are more effective. The low amounts of sinigrin in grated horseradish products (FA, FB) are related to the injuring of the cells during processing and the long time in which hydrolysis of glucosinolates can take place.

5.4.4 SFE-SFC-MS/MS

At this stage it is not possible to show a fully developed SFE-SFC-MS/MS method for the determination of sinigrin, as the time spent at the European headquarter of Shimadzu was to short too overcome all challenges.

One major obstacle was the sensitivity, as for the optimization of the MS and ESI parameters a standard solution of 1 g/L of sinigrin was needed. This means that concentrations of sinigrin have to be higher than 0.1 % in the respective horseradish products. For grated horseradish products it was impossible to find any peak as the concentration was arround 0.007 mg/g fresh weight (see chapter 5.4.3). As root samples show sinigrin concentrations of 3 to 17 mg/g fresh weight (see chapter 5.4.3), these samples were used to optimize the extraction and separation parameters. First tests of roots showed very low signals. So first of all the split ratio was set to 0 (backpressure A = 15, B = 40 MPa), however this lead only to a very small improvement. According to Solana *et al.* [187], polar co-eluants are necessary to extract polar compounds, due to the fact that super critical CO_2 has a non-polar nature. Previous work of this group [188] has shown that in the case of glucosinolates, water as co-eluant gives the highest extraction yield compared to ethanol and methanol. However, in our case the use of water led only to a small increase of the signals and did not show the enormous effect as the group of Solana found. Also variation of the extraction temperature had no significant effect.

In order to improve the separation, an ethylpyridine and C18 intersil column were applied, whereby the C18 column showed the best results. The peak form was still asymmetrically, thus different gradients were tested to solve this problem, namely methanol + 0.1 % trifluoroacetic acid and H_2O:MeOH=1:1 + 0.1 % trifluoroacetic acid as co-solvents. The best results were achieved by using only methanol as co-solvent.

By interpreting the results we had to consider that maybe a massive loss of sinigrin occurred during sample preparation. Furthermore the interpretation of the data is very complicated as the concentration of sinigrin varies strongly within horseradish products. To sum up, SFE-SFC-MS/MS is an interesting tool to measure sinigrin, however the drop of sample preparation is no further an advantage as the procedures needed to inactivate myrosinase and the processes of drying and pulverizing horseradish roots are very time consuming. Further investigations are needed to improve the yield of extraction, for example finding an appropriate co-eluant, and to improve the parameters of separation, such as checking different columns.

6 Conclusion

Four analytical methods, each consisting of a liquid extraction procedure for sample preparation, could be developed and successfully validated in this thesis. The analytical methods include a procedure for the determination of allyl isothiocyanate with GC-FID, one for the measurement of the enzymatic activity of *myrosinase* based on spectrophotometry and two different LC-MS/MS methods for the determination of sinigrin and citric acid, respectively.

For the sonification assisted liquid extraction procedure of allyl isothiocyanate, it could be shown that the use of triethylamine as a displacer with the concentration of 0.5 % in the extraction solvent dichloromethane is a crucial factor in order to receive stable values of the internal standard. With the use of a displacer recoveries of the internal standard of 99 ± 4 % were found. In addition it could be proven that allyl and hexyl isothiocyanates are stable for at least 71 hours. Further, variations of the analyte in subsequent measurements could be ascribed to the inhomogeneous distribution of allyl isothiocyanate within the samples and an influence of the pH could be refuted as the pH ofr six different horseradish products differed only about 4.0 ± 0.2 pH values. A further investigation of the homogeneity of the analyte in fife different brands of grated horseradish products revealed standard deviations from 11 to 63 %, confirming that fibrous products suffer from partially great inhomogeneities. Whereby significant differences between and within the products were found. At last the developed method was used to examine whether there is a difference in the decrease of the pungency between two grated products of different brands after repeated opening over five days. Based on the results, no significant difference could be derived.

In literature, *myrosinase* activity tests differ in the way they use [181] or do not use [8] ascorbate for the photometric test. In our measurements it was not possible to detect any enzyme activity at all in absence of ascorbate. For the analyzed horseradish roots a maximum of the enzyme activity was found at 0.75 mM of ascorbate in the test solution. However, according to Li *et al.* [8] the dependence of the enzyme on the ascorbate concentration differs greatly among horseradish genotypes. Therefore it is recommended to first measure the activity once at a defined concentration of ascorbate and then to additionally evaluate the minimum and maximum activity in dependence of the ascorbate concentration in order to see the overall picture, which allows the comparison of the enzyme activity of different horseradish genuses. Interestingly the determination of the enzyme activity in grated horseradish products revealed no enzyme activity at all. It is thought that metabislufite and its hydrolysis products inhibit the *myrosinase*

activity, as it is the case for sulfate [97]. Here an experimental proof is required to confirm this hypothesis.

For the examination of the amount and distribution of citric acid in two horseradish roots, they were separated in segments in the longitudinal length and into an outer and inner part. The results revealed that in the longitudinal length, there is no significant difference in the concentration, however between the core and the outer part a gradient was observed. The core contained in average 11.4 mg/g fresh weight and the outer part had average values of 6.9 mg/g fresh weight of citric acid. In comparison, the determination of the amount and distribution of sinigrin in two other roots with the same segmentation found values for sinigrin in the outer part of 400 % of the core values. In average the core had about 4 mg/g fresh weight and the outer part around 15 mg/g fresh weight of sinigrin. These results are congruent with the theory that glucosinolates and their break down products have a protective purpose in *brassicaceae* plants. As citric acid has an inhibiting effect on the *myrosinase* activity, high amounts of sinigrin coupled to low amounts of citric acid in the outer part enable an effective defense against pests injuring the outer part of horseradish roots.

Additionally, also the distribution of sinigrin in grated horseradish products was studied. Surprisingly the amount of sinigrin found in grated horseradish products was below 0.007 mg/g fresh weight. This can be explained by a more or less complete hydrolysis of sinigrin, as a consequence of cell injuring during processing. Taking all results together we can infer that after grating horseradish roots, sinigrin is totally hydrolysed, however due to the fact that allyl isothiocyanate is fat soluble, this substance is trapped in the oil which is added during the production. This means that the pungency of grated horseradish products is related to the amount and stability of ally isothiocyanate in these oils. This in fact explains why there was no significant difference in the decrease of the allyl isothiocyanate content in the two analyzed brands, as they further did not differed in the composition of their ingredients. In addition, the reproducible results after intensive blending of the fibers can be explained by a redistribution of the oil and therefore of the allyl isothiocyanate over the product. From the point of view of a manufacturer this means, that the homogeneity of the pungency in a product can be improved, by a method where the oil is homogeneously distributed over the fibers. Thus, the oil needs to be fixed at the surface of the fibers. Moreover, the content of allyl isothiocyanate can be increased by a feedstock with high amounts of sinigrin and a process where the loss of allyl isothiocyanate is minimized, for example by the addition of the oil as quickly as possible after grating and low processing temperatures.

As an outlook the developed methods are ready for use in order to evaluate further influencing parameters on the amount of sinigrin in horseradish roots and on the content of allyl isothiocyanate in different kinds of horseradish products.

7 References

[1] K. Ina, A. Sano, M. Nobukuni, and I. Kishima. Volatile components of wasabi (wasabia japonica) and horseradish (cocholeria aroracia). *Nippon Shokuhin Kogyo Gakkaishi*, 28: 365–370, 1981.

[2] H. Masuda, Y. Harada, M. Nakajima, and H. Tabeta. Characteristic odorants of wasabi (wasabia japonica matsum), japanese horseradish, in comparison with those of horseradish (armoracia rusticana). biotechnology for improved foods and flavours. 637:67–78, 1996.

[3] T. Sultana, G. P. Savage, D. L. McNeil, N. G. Porter, and B. Clark. Comparison of flavour compounds in wasabi and horseradish. *Food, Agriculture & Environment*, 2(1): 117–121, 2003.

[4] L. Tomsone, Z. Kruma, R. Galoburda, and T. Talou. Composition of volatile compounds of horseradish roots (armoracia rusticana l.) depending on the genotype: Lolita tomsone, zanda kruma, ruta galoburda, thierry talou. *Proceedings of the Latvia University of Agriculture*, 29(1):1–10, 2013.

[5] G. R. Fenwick, R. K. Heaney, W. J. Mullin, and C. H. VanEtten. Glucosinolates and their breakdown products in food and food plants. *C R C Critical Reviews in Food Science and Nutrition*, 18(2):123–201, 2009.

[6] K. Grob and P. Matile. Capillary gc of glucosinolate-derived horseradish constituents. *Phytochemistry*, 19(8):1789–1793, 1980.

[7] R. Agneta, C. Möllers, S. De Maria, and A. R. Rivelli. Evaluation of root yield traits and glucosinolate concentration of different armoracia rusticana accessions in basilicata region (southern italy). *Scientia Horticulturae*, 170:249–255, 2014.

[8] X. Li and M. M. Kushad. Correlation of glucosinolate content to myrosinase activity in horseradish (armoracia rusticana). *Journal of agricultural and food chemistry*, 52(23): 6950–6955, 2004.

[9] R. Kosson and M. Horbowicz. Some quality characteristics including isothiocyanates content of horseradish cream as affected by storage period. *Vegetable Crops Research Bulletin*, 71:123–132, 2009.

[10] 27.08.2016 14:58. URL `https://www.plantvillage.org/en/topics/horseradish`.

[11] G. Gärtner, B. Meyer, and J. Scherbius. *Ökonomisch-technische Flora der Wetterau*, volume 3. Guilhauman, Frankfurt am Main, 1802.

[12] Georg Vogel. *Handbuch des speziellen Gemüsebaues*. Ulmer, Stuttgart (Hohenheim), 1996. ISBN 3800152851.

[13] 27.08.2016 17:35. URL `https://www.bmlfuw.gv.at/land/lebensmittel/trad-lebensmittel/spezialkulturen/steirischer_kren.html`.

[14] D. M. Considine and G. D. Considine. *Foods and Food Production Encyclopedia*. Springer US, Boston, MA, 1995. ISBN 146848513X.

[15] 27.08.2016 18:05. URL `http://horticulture.oregonstate.edu/content/horseradish-0`.

[16] 27.08.2016 18.40. URL `http://rustichem.eu/index.php?menu=02-torma`.

[17] R. Bader and M. Kreisel. Gemüseernte 2015: Endgültige ergebnisse. *Statistik Austria*, pages 1–15, 2015.

[18] 28.08.2016 10:46. URL `http://www.feldbacher.at/en/products/horseradish-dip.html`.

[19] F. Oesterlen. *Lehrbuch der Arzneimittellehre*. Druckerei Laupp&Siebeck, Tübingen, 5 edition, 1853.

[20] D. van Eylen, Indrawati, M. Hendrickx, and A. van Loey. Temperature and pressure stability of mustard seed (sinapis alba l.) myrosinase. *Food Chemistry*, 97(2):263–271, 2006.

[21] 28.08.2016 11:48. URL `http://www.avogel.de/pflanzenlexikon/merrettich.php`.

[22] 28.08.2016 12:31. URL `http://www.apotheken.de/club/40225/apotheke-an-der-uni-klinik/leistungen/heilpflanzenlexikon/?hpid=7536&cHash=87dc59de0dab393181c874e807cb99b3`.

[23] C. Fimognari, E. Turrini, L. Ferruzzi, M. Lenzi, and P. Hrelia. Natural isothiocyanates: genotoxic potential versus chemoprevention. *Mutation research*, 750(2):107–131, 2012.

[24] M. E. Cartea and P. Velasco. Glucosinolates in brassica foods: Bioavailability in food and significance for human health. *Phytochemistry Reviews*, 7(2):213–229, 2008.

[25] 28.08.2016 12:46. URL `https://ndb.nal.usda.gov/ndb/foods`.

[26] J.W. Fahey, A.T. Zalcmann, and P. Talalay. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*, 56(1):5–51, 2001.

[27] B. A. Halkier and J. Gershenzon. Biology and biochemistry of glucosinolates. *Annual review of plant biology*, 57:303–333, 2006.

[28] E. Josefsson. Glucosinolate content and amino acid composition of rapeseed(brassica napus) meal as affected by sulphur and nitrogen nutrition. *Journal of the Science of Food and Agriculture*, 21(2):98–103, 1970.

[29] F. J. Zhao, A. P. Wood, and S. P. McGrath. Effects of sulfur nutrition on growth and nitrogen fixation of pea (pisum sativum l.). *Plant and Soil*, 212(2):207–217, 1999.

[30] J. Fieldsend and G. F. J. Milford. Changes in glucosinolates during crop development in single- and double-low genotypes of winter oilseed rape (brassica napus): I. production and distribution in vegetative tissues and developing pods during development and potential role in the recycling of sulphur within the crop. *Annals of Applied Biology*, 124(3):531–542, 1994.

[31] M. Y. Hirai, M. Klein, Y. Fujikawa, M. Yano, D. B. Goodenowe, Y. Yamazaki, S. Kanaya, Y. Nakamura, M. Kitayama, H. Suzuki, N. Sakurai, D. Shibata, J. Tokuhisa, M. Reichelt, J. Gershenzon, J. Papenbrock, and K. Saito. Elucidation of gene-to-gene and metabolite-to-gene networks in arabidopsis by integration of metabolomics and transcriptomics. *The Journal of biological chemistry*, 280(27):25590–25595, 2005.

[32] I. R. Redovnikovic, T. Glicetic, K. Delonga, and J. Vorkapic-Furac. Glucosinolates and their potential role in plant. *Periodicum Biologicum*, 110(4):297–309, 2008.

[33] L. M. Searle, K. Chamerlain, T. Rausch, and D. N. Butcher. The conversion of 3-indolylmethylglucosinolate to 3-indolylacetonitrile by myrosinase, and its relevance to the clubroot disease of the cruciferae. *Journal of Experimental Botany*, 33(5):935–942, 1982.

[34] S. Kawakishi and T. Kaneko. Interaction of proteins with allyl isothiocyanate. *Journal of Agricultural and Food Chemistry*, 35(1):85–88, 1987.

[35] A. A. Agrawal, J. K. Conner, M. T. J. Johnson, and R. Wallsgrove. Ecological genetics of an induced plant defense against herbivores: Additive genetic variance and costs of phenotypic plasticity. 56(11):2006–2213, 2002.

[36] I. Mewis, C. Ulrich, and W. H. Schnitzler. The role of glucosinolates and their hydrolysis products in oviposition and host-plant finding by cabbage webworm, hellula undalis. *Entomologia Experimentalis et Applicata*, 105(2):129–139, 2002.

[37] A. Ratzka, H. Vogel, D. J. Kliebenstein, T. Mitchell-Olds, and J. Kroymann. Disarming the mustard oil bomb. *Proceedings of the National Academy of Sciences of the United States of America*, 99(17):11223–11228, 2002.

[38] U. Wittstock, N. Agerbirk, E. J. Stauber, C. E. Olsen, M. Hippler, T. Mitchell-Olds, J. Gershenzon, and H. Vogel. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences of the United States of America*, 101(14):4859–4864, 2004.

[39] A.M.E. Jones, P. Winge, A. Bones, R. Cole, and J. T. Rossiter. Characterization and evolution of a myrosinase from the cabbage aphid brevicoryne brassicae. *Insect Biochemistry and Molecular Biology*, 32(3):275–284, 2002.

[40] U. Smolinska, M. J. Morra, G. R. Knudsen, and R. L. James. Isothiocyanates produced by brassicaceae species as inhibitors of fusarium oxysporum. *Plant Disease*, 87(4):407–412, 2003.

[41] M. Mari, O. Leoni, R. Iori, and T. Cembali. Antifungal vapour-phase activity of allyl-isothiocyanate against penicillium expansum on pears. *Plant Pathology*, 51(2):231–236, 2002.

[42] Y. Li, G. Kiddle, R. N. Bennett, and R. M. Wallsgrove. Local and systemic changes in glucosinolates in chinese and european cultivars of oilseed rape (brassica napus l.) after inoculation with sclerotinia sclerotiorum (stem rot). *Annals of Applied Biology*, 134(1): 45–58, 1999.

[43] J. A. Kirkegaard, P. A. Gardner, J. F. Angus, and E. Koetz. Effect of brassica break crops on the growth and yield of wheat. *Australian Journal of Agricultural Research*, 45 (3):529, 1994.

[44] J. F. Angus, P. A. Gardner, J. A. Kirkegaard, and J. M. Desmarchelier. Biofumigation: Isothiocyanates released from brassica roots inhibit growth of the take-all fungus. *Plant and Soil*, 162(1):107–112, 1994.

[45] M. Sarwar, J. A. Kirkegaard, P.T.W. Wong, and J. M. Desmarchelier. Biofumigation potential of brassicas: Iii. in vitro toxicity ofisothiocyanates to soil-borne fungal pathogens. *Plant and Soil*, 201(1):103–112, 1998.

[46] H. Ishimoto, Y. Fukushi, T. Yoshida, and S. Tahara. Rhizopus and fusarium are selected as dominant fungal genera in rhizospheres of brassicaceae. *Journal of chemical ecology*, 26(10):2387–2399, 2000.

[47] R. S. Zeng, A. U. Mallik, and E. Setliff. Growth stimulation of ectomycorrhizal fungi by root exudates of brassicaceae plants: Role of degraded compounds of indole glucosinolates. *Journal of chemical ecology*, 29(6):1337–1355, 2003.

[48] A. Kjaer and L. P. Olesen. Non-protein amino acids, cyanogenic glycosides and glucosinolates. *Specialist Periodical Reports*, 2:179–203, 1976.

[49] Z. Xu, L. Escamilla-Trevino, L. Zeng, M. Lalgondar, D. Bevan, B. Winkel, A. Mohamed, C.-L. Cheng, M.-C. Shih, J. Poulton, and A. Esen. Functional genomic analysis of arabidopsis thaliana glycoside hydrolase family 1. *Plant molecular biology*, 55(3):343–367, 2004.

[50] W. P. Burmeister, S. Cottaz, H. Driguez, R. Iori, S. Palmieri, and B. Henrissat. The

crystal structures of sinapis alba myrosinase and a covalent glycosyl–enzyme intermediate provide insights into the substrate recognition and active-site machinery of an s-glycosidase. *Structure*, 5(5):663–676, 1997.

[51] S. Chen and B. A. Halkier. Functional expression and characterization of the myrosinase myr1 from brassica napus in saccharomyces cerevisiae. *Protein expression and purification*, 17(3):414–420, 1999.

[52] R. Bernardi, M. G. Finiguerra, A. A. Rossi, and S. Palmieri. Isolation and biochemical characterization of a basic myrosinase from ripe crambe abyssinica seeds, highly specific for epi-progoitrin. *Journal of agricultural and food chemistry*, 51(9):2737–2744, 2003.

[53] M. Lenman, A. Falk, J Rodin, A.-S. Hoglund, B. Ek, and L. Rask. Differential expression of myrosinase gene families. *Plant Physiology*, 103:703–711, 1993.

[54] A. Bones and J. T. Rossiter. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry*, 67(11):1053–1067, 2006.

[55] E. Engel, C. Baty, D. Le Corre, I. Souchon, and N. Martin. Flavor-active compounds potentially implicated in cooked cauliflower acceptance. *Journal of Agricultural and Food Chemistry*, 50(22):6459–6467, 2002.

[56] Z.-T. Jiang, R. Li, and J. C. Yu. Pungent components from thioglucosides in armoracia rusticana grown in china, obtained by enzymatic hydrolysis. *Food Technol. Biotechnol.*, 44(1):41–45, 2006.

[57] J. Gilbert and H. E. Nursten. Volatile constituents of horseradish roots. *Journal of the Science of Food and Agriculture*, 23(4):527–539, 1972.

[58] 13.09.2016 15:00. URL `http://www.pherobase.com`.

[59] H. E. van Doorn, G. C. van der Kruk, G.-J. van Holst, Natasja C. M. Raaijmakers-Ruijs, E. Postma, B. Groeneweg and W. H. Jongen. The glucosinolates sinigrin and progoitrin are important determinants for taste preference and bitterness of brussels sprouts. *Journal of the Science of Food and Agriculture*, 78:30–38, 1998.

[60] H.-W. Chin and R. C. Lindsay. Volatile sulfur compounds formed in disrupted tissues of different cabbage cultivars. *Journal of Food Science*, 58(4):835–839, 1993.

[61] F. Viana. Chemosensory properties of the trigeminal system. *ACS chemical neuroscience*, 2(1):38–50, 2011.

[62] B. Lüthy and P. Matile. The mustard oil bomb: Rectified analysis of the subcellular organisation of the myrosinase system. *Biochemie und Physiologie der Pflanzen*, 179(1-2): 5–12, 1984.

[63] A. Bones and J. T. Rossiter. The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum*, 97(1):194–208, 1996.

[64] L. Guignard. Recherches sur la localisation des principes actifs des cruciferes. *Journal de botanique*, 22(4):385–395, 1890.

[65] W. Spatzier. Überdas aufreten und die physiologische bedeutung des myrosins in der pflanze. *Jahrbücher für Wissenschaftliche Botanik*, 25:39–78, 1893.

[66] O. P. Thangstad, K. Evjen, and A. Bones. Immunogold-em localization of myrosinase in brassicaceae. *Protoplasma*, 161(2-3):85–93, 1991.

[67] J. G. Vaughan, A. J. MacLeod, and B. M. Jones. *The biology and chemistry of the Cruciferae: Properties and function of plant myrosinases*. Academic Press, London and New York, 1976. ISBN 9780127151502.

[68] A. Bones, O. P. Thangstad, O. A. Haugen, and T. Espevik. Characterization of monoclonal antibodies against myrosinase. *Journal of Experimental Botany*, 42(245):1541–1550, 1991.

[69] P. J. Kelly, A. Bones, and J. T. Rossiter. Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of brassica juncea. *Planta*, 206(3):370–377, 1998.

[70] O. A. Koroleva, A. Davies, R. Deeken, M. R. Thorpe, A. D. Tomos, and R. Hedrich. Identification of a new glucosinolate-rich cell type in arabidopsis flower stalk. *Plant Physiology*, 124(2):599–608, 2000.

[71] K. Grob and P. H. Matile. Vacuolar location of glucosinolates in horseradish root cells. *Plant Science Letters*, 14(4):327–335, 1979.

[72] R. Kissen, J. T. Rossiter, and A. Bones. The 'mustard oil bomb': not so easy to assemble?! localization, expression and distribution of the components of the myrosinase enzyme system. *Phytochemistry*, 8(1):69–86, 2009.

[73] F. S. Hanschen, E. Lamy, M. Schreiner, and S. Rohn. Reactivity and stability of glucosinolates and their breakdown products in foods. *Angewandte Chemie International Edition*, 53(43):11430–11450, 2014.

[74] Z. Zhang, J. A. Ober, and D. J. Kliebenstein. The gene controlling the quantitative trait locus epithiospecifier modifier1 alters glucosinolate hydrolysis and insect resistance in arabidopsis. *The Plant cell*, 18(6):1524–1536, 2006.

[75] U. Wittstock and M. Burow. Glucosinolate breakdown in arabidopsis: mechanism, regulation and biological significance. *The Arabidopsis book / American Society of Plant Biologists*, 8:e0134, 2010.

[76] M. Burow and U. Wittstock. Regulation and function of specifier proteins in plants. *Phytochemistry Reviews*, 8(1):87–99, 2009.

[77] V. Gil and A. J. MacLeod. The effects of ph on glucosinolate degradation by a thioglucoside glucohydrolase preparation. *Phytochemistry*, 19(12):2547–2551, 1980.

[78] C. H. VanEtten, M. E. Daxenbichler, J. E. Peters, and H. L. Tooke. Variation in enzymatic degradation products from the major thioglucosides in crambe abyssinica and brassica napus seed meals. *Journal of Agricultural and Food Chemistry*, 14(4):426–430, 1966.

[79] Y. Uda, T. Kurata, and N. Arakawa. Effects of thiol compounds on the formation of nitriles from glucosinolates. *Agricultural and Biological Chemistry*, 50(11):2741–2746, 1986.

[80] M. Ohtsuru, I. Tsuruo, and T. Hata. Studies on fungous myrosinase. 4. production and stability of intracellular myrosinase from aspergillus niger. *Agricultural and Biological Chemistry*, 37:967–971, 1973.

[81] F. Francis, G. Lognay, J.-P. Wathelet, and E. Haubruge. Characterisation of aphid myrosinase and degradation studies of glucosinolates. *Archives of insect biochemistry and physiology*, 50(4):173–182, 2002.

[82] W. P. Burmeister, S. Cottaz, P. Rollin, A. Vasella, and B. Henrissat. High resolution x-ray crystallography shows that ascorbate is a cofactor for myrosinase and substitutes for the function of the catalytic base. *The Journal of biological chemistry*, 275(50):39385–39393, 2000.

[83] A. Bones and J. T. Rossiter. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry*, 67(11):1053–1067, 2006.

[84] D. T. Verhoeven, H. Verhagen, R. A. Goldbohm, van den Brandt, P. A., and G. van Poppel. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chemico-Biological Interactions*, 103(2):79–129, 1997.

[85] H. Sidransky, N. Ito, and E. Verney. Influence of alpha-naphthyl-isothiocyanate on liver tumorigenesis in rats ingesting ethionine and n-2-fluorenylacetamide. *Journal of the National Cancer Institute*, 37(5):677–686, 1966.

[86] M. Vermorel, R. K. Heaney, and G. R. Fenwick. Antinutritional effects of the rapeseed meals, darmor and jet neuf, and progoitrin together with myrosinase, in the growing rat. *Journal of the Science of Food and Agriculture*, 44(4):321–334, 1988.

[87] D. W. Griffiths, J. E. Bradshaw, J. Taylor, and D. J. Gemmell. Effect of cultivar and harvest date on the glucosinolate and s-methylcysteine sulphoxide content of swedes (brassica napus ssp rapifera). *Journal of the Science of Food and Agriculture*, 56(4): 539–549, 1991.

[88] E. A. S. Rosa, R. K. Heaney, C. A. M. Portas, and G. R. Fenwick. Changes in glucosinolate

concentrations inbrassicacrops (b oleraceaandb napus)throughout growing seasons. *Journal of the Science of Food and Agriculture*, 71(2):237–244, 1996.

[89] A. J. MacLeod and H. E. Pikk. A comparison of the chemical flavour composition of some brussels sprouts cultivars grown at different crop spacings. *Phytochemistry*, 17(6): 1029–1032, 1978.

[90] R. P. Bodnaryk. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry*, 31(8):2671–2677, 1992.

[91] R. P. Bodnaryk and P. Palaniswamy. Glucosinolate levels in cotyledons of mustard,brassica juncea l. and rape,b. napus l. do not determine feeding rates of flea beetle,phyllotreta cruciferae (goeze). *Journal of chemical ecology*, 16(9):2735–2746, 1990.

[92] R. P. Bodnaryk. Developmental profile of sinalbin (p-hydroxybenzyl glucosinolate) in mustard seedlings,sinapis alba l., and its relationship to insect resistance. *Journal of chemical ecology*, 17(8):1543–1556, 1991.

[93] E. Ciska, B. Martyniak-Przybyszewska, and H. Kozlowska. Content of glucosinolates in cruciferous vegetables grown at the same site for two years under different climatic conditions. *Journal of Agricultural and Food Chemistry*, 48(7):2862–2867, 2000.

[94] G. G. Freeman and N. Mossadeghi. Studies on relationship between water regime and flavour strength in watercress (rorippa nasturtium-aquaticum (l) hayek), cabbage (brassica oleracea capitata) and onion (allium cepa). *Journal of Horticultural Science*, 48 (4):365–378, 2015.

[95] R. P. Bodnaryk. Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry*, 35(2):301–305, 1994.

[96] L. Song and P. J. Thornalley. Effect of storage, processing and cooking on glucosinolate content of brassica vegetables. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 45(2):216–224, 2007.

[97] M. Shikita, J. W. Fahey, T. R. Golden, W. D. Holtzclaw, and P. Talalay. An unusual case of 'uncompetitive activation' by ascorbic acid: Purification and kinetic properties of a myrosinase from raphanus sativus seedlings. *Biochemical Journal*, 341(3):725–732, 1999.

[98] A. Smiechowska, A. Bartoszek, and J. Namiesnik. Determination of glucosinolates and their decomposition products—indoles and isothiocyanates in cruciferous vegetables. *Critical Reviews in Analytical Chemistry*, 40(3):202–216, 2010.

[99] R. K. Heaney, E. A. Spinks, and G. R. Fenwick. Improved method for the determination of the total glucosinolate content of rapeseed by determination of enzymically released glucose. *The Analyst*, 113(10):1515–1518, 1988.

[100] C. H. VanEtten, C. E. McGrew, and M. E. Daxenbichler. Glucosinolate determination in cruciferous seeds and meals by measurement of enzymatically released glucose. *Journal of Agricultural and Food Chemistry*, 3(22):483–487, 1974.

[101] W. Thies. Determination of the glucosinolate content in commercial rapeseed loads with a pocket reflectometer. *Fette, Seifen, Anstrichmittel*, 87(9):347–350, 1985.

[102] H.-J. Fiebig, A. Sendfeld, M. Jörden, and K. Aitzetmüller. Untersuchungen zur bestimmung des gesamtglucosinolatgehaltes von rapssamen. *Fett Wissenschaft Technologie/Fat Science Technology*, 91(7):266–271, 1989.

[103] A. Sendfeld, H.-J. Fiebig, and K. Aitzetmüller. Bestimmung des gesamtglucosinolatgehaltes von rapssamen durch ionenchromatographie von enzymatisch freigesetztem sulfat. *Fett Wissenschaft Technologie/Fat Science Technology*, 90(3):83–85, 1988.

[104] M. M. Choi, M. M. Liang, and A. W. Lee. A biosensing method with enzyme-immobilized eggshell membranes for determination of total glucosinolates in vegetables. *Enzyme and Microbial Technology*, 36(1):91–99, 2005.

[105] E. Schnug. The certified glucosinolate content in three rapeseeds (crms 19, 366 and 367). *Fett Wissenschaft Technologie/Fat Science Technology*, (89):438–442, 1987.

[106] H. E. van Doorn, G.-J. van Holst, G. C. van der Kruk, N. C. Raaijmakers-Ruijs, and E. Postma. Quantitative determination of the glucosinolates sinigrin and progoitrin by specific antibody elisa assays in brussels sprouts. *Journal of Agricultural and Food Chemistry*, 46(2):793–800, 1998.

[107] W. Thies. Photometric determination of indolyl glucosinolates in seeds of rapeseed. *Fett Wissenschaft Technologie/Fat Science Technology*, 92(4):161–164, 1990.

[108] R. Koprna, O. Kolovrat, and P. Nerusil. Comparison of accuracy of screening methods for determination of glucosinolate content in winter rape seed. *Oilseed crops*, (2):267–274, 2002.

[109] T. Furuya. Gas-liquid chromatography of plant glycosides. *Journal of Chromatography A*, 18:152–156, 1965.

[110] E. W. Underhill and D. F. Kirkland. Gas chromatography of trimethylsilyl derivatives of glucosinolates. *Journal of Chromatography A*, 57:47–54, 1971.

[111] E. W. Underhill and D. F. Kirkland. l-2-amino-4-phenylbutyric acid and 2-phenylethylglucosinolate, precursors of 2-hydroxy-2-phenylethylglucosinolate. *Phytochemistry*, 11(6):1973–1979, 1972.

[112] W. Thies. Quantitative gas liquid chromatography of glucosinolates on a microliter scale. *Fette, Seifen, Anstrichmittel*, 78(6):231–234, 1976.

[113] R. K. Heaney and G. R. Fenwick. The analysis of glucosinolates in brassica species using gas chromatography. direct determination of the thiocyanate ion precursors, glucobrassicin and neoglucobrassicin. *Journal of the Science of Food and Agriculture*, 31(6):593–599, 1980.

[114] O. Olsen and H. Soerensen. Sinalbin and other glucosinolates in seeds of double low rape species and brassica napus cv bronowski. *Journal of Agricultural and Food Chemistry*, 28 (1):43–48, 1980.

[115] B. W. Chistensen, A. Kjær, J. O. Madsen, C. E. Olsen, O. Olsen, and H. Sorensen. Mass-spectrometric characteristics of some pertrimethyl-silylated desulfoglucosinolates. *Tetrahedron*, 38(3):353–357, 1982.

[116] B. A. Slominski and L. D. Campbell. Gas chromatographic determination of indole glucosinolates—a re-examination. *Journal of the Science of Food and Agriculture*, 40(2): 131–143, 1987.

[117] M. M. Kushad, A. F. Brown, A. C. Kurilich, J. A. Juvik, B. P. Klein, M. A. Wallig, and E. H. Jeffery. Variation of glucosinolates in vegetable crops of brassicaoleracea. *Journal of Agricultural and Food Chemistry*, 47(4):1541–1548, 1999.

[118] R. S. Malabed, M. G. Noel, B. C. Aton, and E. A. Toribio. Characterization of the glucosinolates and isothiocyanates in mustard (brassica juncea l.) extracts and determination of its myrosinase activity and antioxidant capacity. *De La Salle University, Manila, Philippines*, pages 1–7, 2014.

[119] M.-K. Lee, M. V. Arasu, J.-H. Chun, J. M. Seo, K.-T. Lee, S.-T. Hong, I. H. Kim, Y.-H. Lee, Y.-S. Jang, and S.-J. Kim. Identification and quantification of glucosinolates in rapeseed (brassica napus l.) sprouts cultivated under dark and light conditions. *Korean Journal of Environmental Agriculture*, 32(4):315–322, 2013.

[120] B. Matthäus and H. Luftmann. Glucosinolates in members of the family brassicaceae: Separation and identification by lc/esi-ms-ms. *Journal of Agricultural and Food Chemistry*, 48(6):2234–2239, 2000.

[121] N. Kaushik and A. Agnihotri. High-performance liquid chromatographic method for separation and quantification of intact glucosinolates. *Chromatographia*, 50(1-2):128, 1999.

[122] F. A. Mellon, R. N. Bennett, B. Holst, and G. Williamson. Intact glucosinolate analysis in plant extracts by programmed cone voltage electrospray lc/ms: performance and comparison with lc/ms/ms methods. *Analytical biochemistry*, 306(1):83–91, 2002.

[123] J. Volden, G. I. Borge, G. B. Bengtsson, M. Hansen, I. E. Thygesen, and T. Wicklund. Effect of thermal treatment on glucosinolates and antioxidant-related parameters in red

cabbage (brassica oleracea l. ssp. capitata f. rubra). *Food Chemistry*, 109(3):595–605, 2008.

[124] L. Song, J. J. Morrison, N. P. Botting, and P. J. Thornalley. Analysis of glucosinolates, isothiocyanates, and amine degradation products in vegetable extracts and blood plasma by lc-ms/ms. *Analytical biochemistry*, 347(2):234–243, 2005.

[125] Q. Tian, R. A. Rosselot, and S. J. Schwartz. Quantitative determination of intact glucosinolates in broccoli, broccoli sprouts, brussels sprouts, and cauliflower by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. *Analytical biochemistry*, 343(1):93–99, 2005.

[126] M. J. Cwika, H. Wua, M. Muzzioa, D. L. McCormicka, and I. Kapetanovic. Direct quantitation of glucoraphanin in dog and rat plasma by lc-ms/ms. *Journal of pharmaceutical and biomedical analysis*, 52(4):544–549, 2010.

[127] J. K. Troyer, K. K. Stephenson, and J. W. Fahey. Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography. *Journal of Chromatography A*, 919(2):299–304, 2001.

[128] K. L. Wade, I. J. Garrard, and J. W. Fahey. Improved hydrophilic interaction chromatography method for the identification and quantification of glucosinolates. *Journal of chromatography. A*, 1154(1-2):469–472, 2007.

[129] B. Buszewski and S. Noga. Hydrophilic interaction liquid chromatography (hilic) - a powerful separation technique. *Analytical and bioanalytical chemistry*, 402(1):231–247, 2012.

[130] L. West, I. Tsui, and G. Haas. Single column approach for the liquid chromatographic separation of polar and non-polar glucosinolates from broccoli sprouts and seeds. *Journal of Chromatography A*, 966(1-2):227–232, 2002.

[131] G. Sivakumar, A. Aliboni, and L. Bacchetta. Hplc screening of anti-cancer sulforaphane from important european brassica species. *Food Chemistry*, 104(4):1761–1764, 2007.

[132] W. C. Chiang, D. J. Pusateri, and R. E. Leitz. Gas chromatography/mass spectrometry method for the determination of sulforaphane and sulforaphane nitrile in broccoli. *Journal of Agricultural and Food Chemistry*, 46(3):1018–1021, 1998.

[133] K. A. Korb and G. W. Chism. A rapid method for determining allylisothiocyanate in horseradish-containing products. *Journal of Food Science*, 54(3):778–779, 1989.

[134] A. Peruga, J. Beltran, F. Lopez, and F. Hernandez. Determination of methylisothiocyanate in soil and water by hs-spme followed by gc-ms-ms with a triple quadrupole. *Analytical and bioanalytical chemistry*, 406(22):5271–5282, 2014.

[135] A. Jain, K. Reddy-Noone, A. K. Pillai, and K. K. Verma. Conversion to isothiocyanates via dithiocarbamates for the determination of aromatic primary amines by headspace-solid phase microextraction and gas chromatography. *Analytica chimica acta*, 801:48–58, 2013.

[136] M.-R. Marton and V. Lavric. A simple method for the quantification of isothiocyanates from mustard. *U.P.B. Sci. Bull.*, 1(75):63–72, 2013.

[137] J. Li, B. Xie, S. Yan, H. Li, and Q. Wang. Extraction and determination of 4-methylthio-3-butenyl isothiocyanate in chinese radish (raphanus sativus l.) roots. *LWT - Food Science and Technology*, 60(2):1080–1087, 2015.

[138] K. Okano, J. Asano, and G. Ishii. A rapid method for determining the pungent principle in root of japanese radish (raphanus sativus l.). *Journal of the Japanese Society for Horticultural Science*, 59(3):545–550, 1990.

[139] Y. Zhang, C.-G. Cho, G. H. Posner, and P. Talalay. Spectroscopic quantitation of organic isothiocyanates by cyclocondensation with vicinal dithiols. *Analytical biochemistry*, 205 (1):100–107, 1992.

[140] N. Agerbirk, G. R. de Nicola, C. E. Olsen, C. Müller, and R. Iori. Derivatization of isothiocyanates and their reactive adducts for chromatographic analysis. *Phytochemistry*, 118:109–115, 2015.

[141] D. Bertelli, M. Plessi, D. Braghiroli, and A. Monzani. Separation by solid phase extraction and quantification by reverse phase hplc of sulforaphane in broccoli. *Food Chemistry*, 63 (3):417–421, 1998.

[142] K. Nakagawa, T. Umeda, O. Higuchi, T. Tsuzuki, T. Suzuki, and T. Miyazawa. Evaporative light-scattering analysis of sulforaphane in broccoli samples: Quality of broccoli products regarding sulforaphane contents. *Journal of agricultural and food chemistry*, 54 (7):2479–2483, 2006.

[143] R. Scherer, A. C. Rybka, C. A. Ballus, A. D. Meinhart, J. T. Filho, and H. T. Godoy. Validation of a hplc method for simultaneous determination of main organic acids in fruits and juices. *Food Chemistry*, 135(1):150–154, 2012.

[144] K. Herrmann. Über das vorkommen der wesentlichen organischen säuren in gemüse, kartoffeln und getreide. *Z Lebensm Unters Forch (Zeitschrift für Lebensmittel-Untersuchung und -Forschung)*, 155(4):220–233, 1974.

[145] H. S. Lee. Hplc method for separation and determination of nonvolatile organic acids in orange juice. *Journal of Agricultural and Food Chemistry*, 41(11):1991–1993, 1993.

[146] M. H. Suarez, E. R. Rodriguez, and C. D. Romero. Analysis of organic acid content in cultivars of tomato harvested in tenerife. *European Food Research and Technology*, 226 (3):423–435, 2008.

[147] H.-D. Belitz, W. Grosch, and P. Schieberle. *Lehrbuch der Lebensmittelchemie*. Springer-Lehrbuch. Springer, Berlin, Heidelberg, 6., vollst. überarb. aufl. edition, 2008. ISBN 9783540732013.

[148] L. Saavedra and C. Barbas. Validated capillary electrophoresis method for small-anions measurement in wines. *Electrophoresis*, 24(12-13):2235–2243, 2003.

[149] T.A. Bennet-Clark. The role of the organic acids in plant metabolism: Part iii. *New Phytologist*, 32(3):197–230, 1933.

[150] M. Bellion, M. Courbot, C. Jacob, D. Blaudez, and M. Chalot. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS microbiology letters*, 254(2):173–181, 2006.

[151] J. McCluskey, L. Herdman, and K. R. Skene. Iron deficiency induces changes in metabolism of citrate in lateral roots and cluster roots of lupinus albus. *Physiologia Plantarum*, 121 (4):586–594, 2004.

[152] I. Mato, S. Suarez-Luque, and J. F. Huidobro. A review of the analytical methods to determine organic acids in grape juices and wines. *Food Research International*, 38(10): 1175–1188, 2005.

[153] H. U. Bergmeyer and J. Bergmeyer. *Tri- and dicarboxylic acids, purines, pyrimidines and derivatives, coenzymes, inorganic compounds*, volume 2 of *Methods of enzymatic analysis Metabolites*. VCH, Weinheim, 3. ed. edition, 1985. ISBN 0895732378.

[154] A. G. Giumanini, G. Verardo, D. Della Martina, and N. Toniutti. Improved method for the analysis of organic acids and new derivatization of alcohols in complex natural aqueous matrixes: Application to wine and apple vinegar. *Journal of Agricultural and Food Chemistry*, 49(6):2875–2882, 2001.

[155] G. N. Jham, S. A. Fernandes, C. F. Garcia, and A. A. da Silva. Comparison of gc and hplc for the quantification of organic acids in coffee. *Phytochemical analysis : PCA*, 13 (2):99–104, 2002.

[156] I. Mato, S. Suarez-Luque, and J. F. Huidobro. Simple determination of main organic acids in grape juice and wine by using capillary zone electrophoresis with direct uv detection. *Food Chemistry*, 102(1):104–112, 2007.

[157] P. Valentao, G. Lopes, M. Valente, P. Barbosa, P. B. Andrade, B. M. Silva, P. Baptista, and R. M. Seabra. Quantitation of nine organic acids in wild mushrooms. *Journal of agricultural and food chemistry*, 53(9):3626–3630, 2005.

[158] B. M. Silva, P. B. Andrade, G. C. Mendes, R. M. Seabra, and M. A. Ferreira. Study of the organic acids composition of quince (cydonia oblonga miller) fruit and jam. *Journal of Agricultural and Food Chemistry*, 50(8):2313–2317, 2002.

[159] A. R.-B. de Quiros, M. A. Lage-Yusty, and J. Lopez-Hernandez. Hplc analysis of organic acids using a novel stationary phase. *Talanta*, 78(2):643–646, 2009.

[160] I. Molnar-Perl. Simultaneous quantitation of acids and sugars by chromatography: Gas or high-performance liquid chromatography? *Journal of Chromatography A*, 845(1-2): 181–195, 1999.

[161] Y.-J. Zheng, Y.-T. Duan, Y.-F. Zhang, Q.-H. Pan, J.-M. Li, and W.-D. Huang. Determination of organic acids in red wine and must on only one rp-lc-column directly after sample dilution and filtration. *Chromatographia*, 69(11-12):1391–1395, 2009.

[162] T. Perez-Ruiz, C. Maritinez-Lozano, V. Tomas, and J. Martin. High-performance liquid chromatographic separation and quantification of citric, lactic, malic, oxalic and tartaric acids using a post-column photochemical reaction and chemiluminescence detection. *Journal of Chromatography A*, 1026(1-2):57–64, 2004.

[163] M. Wang, F. Qu, X.-Q. Shan, and J.-M. Lin. Development and optimization of a method for the analysis of low-molecular-mass organic acids in plants by capillary electrophoresis with indirect uv detection. *Journal of Chromatography A*, 989(2):285–292, 2003.

[164] A. Kotani, Y. Miyaguchi, E. Tomita, K. Takamura, and F. Kusu. Determination of organic acids by high-performance liquid chromatography with electrochemical detection during wine brewing. *Journal of agricultural and food chemistry*, 52(6):1440–1444, 2004.

[165] V. Magne, M. Mathlouthi, and B. Robilland. Determination of some organic acids and inorganic anions in beet sugar by ionic hplc. *Food Chemistry*, 61(4):449–453, 1998.

[166] C. J. Scarlata and D. A. Hyman. Development and validation of a fast high pressure liquid chromatography method for the analysis of lignocellulosic biomass hydrolysis and fermentation products. *Journal of chromatography. A*, 1217(14):2082–2087, 2010.

[167] P. Flores, P. Hellin, and J. Fenoll. Determination of organic acids in fruits and vegetables by liquid chromatography with tandem-mass spectrometry. *Food Chemistry*, 132(2): 1049–1054, 2012.

[168] R. Fernandez-Fernandez, J. C. Lopez-Martinez, R. Romero-Gonzalez, J. L. Martinez-Vidal, M. I. Alarcón Flores, and A. G. Frenich. Simple lc–ms determination of citric and malic acids in fruits and vegetables. *Chromatographia*, 72(1-2):55–62, 2010.

[169] J. Erro, A. M. Zamarreno, J.-C. Yvin, and J. M. Garcia-Mina. Determination of organic acids in tissues and exudates of maize, lupin, and chickpea by high-performance liquid chromatography-tandem mass spectrometry. *Journal of agricultural and food chemistry*, 57(10):4004–4010, 2009.

[170] J. Qiu and X. Jin. Development and optimization of organic acid analysis in tobacco with

ion chromatography and suppressed conductivity detection. *Journal of Chromatography A*, 950(1-2):81–88, 2002.

[171] Y. Hernandez, M. G. Lobo, and M. Gonzalez. Factors affecting sample extraction in the liquid chromatographic determination of organic acids in papaya and pineapple. *Food Chemistry*, 114(2):734–741, 2009.

[172] M. Castellari, A. Versari, U. Spinabelli, S. Galassi, and A. Amati. An improved hplc method for the analysis of organic acids, carbohydrates, and alcohols in grape musts and wines. *Journal of Liquid Chromatography & Related Technologies*, 23(13):2047–2056, 2000.

[173] S. H. Ashoor and M. J. Knox. Determination of organic acids in foods by high-performance liquid chromatography. *Journal of Chromatography A*, 299:288–292, 1984.

[174] X. Li and M. M. Kushad. Purification and characterization of myrosinase from horseradish (armoracia rusticana) roots. *Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale*, 43(6):503–511, 2005.

[175] 12.09.2016 11:44. URL `http://www.periodensystem-online.de/index.php?id=lists&form=Dichtewerte&sst=21`.

[176] B. Neidhart and W. Wegscheider. *Quality in chemical measurements: Training concepts and teaching materials*. Springer, Berlin and New York, 2001. ISBN 3642630162.

[177] M. G. Ettlinger, G. P. Dateo, B. W. Harrison, T. J. Mabry, and C. P. Thompson. Vitamine c as a coenzyme: The hydrolysis of mustard oil glucosides. *Proceedings of the National Academy of Sciences of the United States of America*, 47:1875–1880, 1961.

[178] J. Li, Q. Wang, Q. Zhou, Y. Sun, and M. Xie. Properties of myrosinase from radish. *Shipin Kexue*, 29(11):451–455, 2008.

[179] A. P. Wilkinson, M. J. Rhodes and R. G. Fenwick. Myrosinase activity of cruciferous vegetables. *Journal of the Science of Food and Agriculture*, 35:543–552, 1984.

[180] J. R. Phelan and J. G. Vaughan. Myrosinase in sinapis alba l. *Journal of Experimental Botany*, 31(5):1425–1433, 1980.

[181] N. Bellostas, I. L. Petersen, J. C. Sorensen, and H. Sorensen. A fast and gentle method for the isolation of myrosinase complexes from brassicaceous seeds. *Journal of biochemical and biophysical methods*, 70(6):918–925, 2008.

[182] T. M. Annesley. Ion suppression in mass spectrometry. *Clinical Chemistry*, 49(7): 1041–1044, 2003.

[183] C. R. Mallet, Z. Lu, and J. R. Mazzeo. A study of ion suppression effects in electrospray

ionization from mobile phase additives and solid-phase extracts. *Rapid communications in mass spectrometry : RCM*, 18(1):49–58, 2004.

[184] J.-P. Antignac, K. de Wasch, F. Monteau, H. de Brabander, F. Andre, and B. Le Bizec. The ion suppression phenomenon in liquid chromatography–mass spectrometry and its consequences in the field of residue analysis. *Analytica chimica acta*, 529(1-2):129–136, 2005.

[185] D. A. Volmer and L. L. Jessome. Ion suppression: A major concern in mass specrometry. *LCGLC North America*, 24(5):498–510, 2006.

[186] R. Pohloudek-Fabini. *Studien über die Chemie und Physiologie der Citronensäure*. VEB Verlag Technik, Berlin, 1955.

[187] M. Solana, S. Mirofci, and A. Bertucco. Production of phenolic and glucosinolate extracts from rocket salad by supercritical fluid extraction: Process design and cost benefits analysis. *Journal of Food Engineering*, 168:35–41, 2016.

[188] M. Solana, I. Boschiero, S. Dall'Acqua, and A. Bertucco. Extraction of bioactive enriched fractions from eruca sativa leaves by supercritical co2 technology using different co-solvents. *The Journal of Supercritical Fluids*, 94:245–251, 2014.

8 List of Figures

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10 Appendix

10.1 List of Chemicals

Chemical	Supplier	CAS-Nr.	Batch-Nr.	%	Amount
Allyl isothiocyanate	Sigma Aldrich	57-06-7	MKBS7089V	95	100 mL
Ascorbinsäure	Sigma Aldrich	50-81-7	BCBP5310V	$\geq$ 99	250 g
Citric acid monohydrate	Sigma Aldrich	5949-29-1	SZBE350AV	99.5-100.5	1 kg
Dichloromethane	Roth	75-09-2		$\geq$ 99,9	2.5 L
DL-Isocitric acid trisodium salt hydrate	Sigma Aldrich	1637-73-6	03501BY	98	5 g
Ethyl acetate	Promochem LGC	141-78-6		99	4 L
Formic acid	Roth	64-18-6	132180744	$\geq$ 98	1 L
Hexyl isothiocyanate	Sigma Aldrich	4404-45-9	MKBB0685V	95	5 g
Kalium-Sinigrin	Tokyo Chemical Industry	3952-98-5	86GUJ-JN		100 mg
KH_2PO_4	Merck	7778-77-0	929A434173	99	1 kg
Methanol lc-ms grade	ChemLab	67-56-1			
Methanol lc-ms grade	VWR ProLabo BPH	67-56-1			
NaOH	Roth	1310-73-2	124211335	> 99	
Pentane	Promochem LGC	109-66-0		98	4L
Toluene	Promochem LGC	108-88-3		99.8	4 L
Tricarballylic acid	Alfa Aesar	99-14-9	W11AO29	98	5 g
Triethylamine	Sigma Aldrich	121-44-8	73-962-3	99+	50 g
Trifluoroacetic acid	Sigma Aldrich	76-05-1	STBF2529V	99	100 mL
Syn. Air	Linde			KW frei	
H_2	Linde	1333-74-0		5.0	
He	Linde	7440-59-7		5.0	
N_2	Linde	7727-37-9		5.0	