Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Glucosinolates in *Lepidium campestre*

– Method development and analysis

Glukosinolater i *Lepidium campestre*

– Metodutveckling och analysering

Michaela Isoz

Department of Molecular Sciences
Master´s thesis • 30 hec • Second cycle, A2E
Agriculture Programme - Food Science
Molecular Sciences, 2018:2
Uppsala 2018

Glucosinolates in *Lepidium campestre* – Method development and analysis

Glukosinolater i *Lepidium campestre* – Metodutveckling och analysering

Michaela Isoz

Supervisor:	Annica Andersson, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Assistant Supervisor:	Lena Dimberg, University of Agricultural Sciences, Department of Molecular Sciences
Examiner:	Roger Andersson, University of Agricultural Sciences, Department of Molecular Sciences

Credits: 30 hec
Level: Second cycle, A2E
Course title: Independent project/degree project in Food Science - Master's thesis
Course code: EX0425
Programme/education: Agriculture Programme - Food Science

Place of publication: Uppsala
Year of publication: 2018
Title of series: Molecular Sciences
Part number: 2018:2
Online publication: http://stud.epsilon.slu.se

Keywords: *Lepidium campestre*, Glucosinolates, Sinigrin, Sulfatase, Sinalbin

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Department of Molecular Sciences

Abstract

Field cress (*Lepidium campestre)* is today not a domesticated crop for public consumption. *Lepidium campestre* is a relative to rapeseed (*Brassica napus)* and both are producing oil. *Lepidium campestre* contain glucosinolates which makes it unattractive for food and feed, however, it has some other properties that makes it attractive in a plant culture point of view. Field cress has a better cold tolerance than rapeseed which means that it can be grown further north. Moreover, *Lepidium campestre* is a perennial crop that can help minimise tillage and therefore help to minimise nitrogen leaching. Glucosinolates is a β-thioglucoside-N-hydroxysulfates that exist in the whole plant but, it is most concentrated in the seeds. Today we know about 130 glucosinolates that are categorised by both structure and the amino acids. Glucosinolates are by itself not toxic, however if the plant is damaged and glucosinolates reacts with the enzyme myrosinase then several products are produced which could be toxic, some studies have shown toxicity in mice and rats. In this study, a method based on an earlier method was modified to be able to analyse glucosinolates in *Lepidium campestre* in a cheaper and faster way. This was done by trial and error testing using the results from the tests and modifying the method after that. Two important steps for making the analysis successful was to use a sinigrin calibration curve and a purified sulfatase. The results from the *Lepidium campestre* analysis showed that one glucosinolate was the dominant one in all the samples which is most likely sinalbin. Twenty-two different *Lepidium campestre* seed samples from three different countries were analysed for the glucosinolate content. The result showed that the content of glucosinolates varied between 180 and 360 μmol/g in all samples except one from Sweden which had a content of almost 600 μmol/g. The mean value for the samples from Sweden was 289 μmol/g, from Germany 273 μmol/g and from USA 292 μmol/g. There was no significant different in glucosinolate content between samples from different countries.

Keywords: Lepidium campestre, Field cress, Glucosinolates, Sinigrin, Sulfatase, Sinalbin

Sammanfattning

Fältkrassing (*Lepidium campestre*) är idag en vildgröda som inte finns för allmänhetens konsumtion. *Lepidium campestre* är en familjemedlem till raps (*Brassica napus*) och båda producerar olja, men *Lepidium campestre* har vissa egenskaper som gör den oattraktiv som mat och foder men samtidigt har den vissa egenskaper som gör den attraktiv ur en växtodlings synvinkel. *L. campestre* har bättre köldtolerans än raps, vilket innebär att det kan odlas längre norrut. Dessutom är *Lepidium campestre* en flerårig gröda som kan bidra till minskad jordbearbetning och därför bidra till att minimera kväveutlakningen. Glukosinolat är en β-tioglukosid-N-hydroxysulfat som finns i hela växten men det är mest koncentrerad i fröerna. Idag känner vi till cirka 130 glukosinolater som kategoriseras utifrån både struktur och aminosyror. Glukosinolater är i sig inte giftiga, men om växten är skadad och glukosinolater reagerar med enzymet myrosinas, produceras flera produkter som kan vara giftiga, vissa studier har visat toxicitet hos möss och råttor.
I denna studie modifierades en metod som baserades på en tidigare metod för att kunna analysera glukosinolater i *Lepidium campestre* på ett snabbare och billigare sätt. Detta lades upp så att man testade metoden för att sedan modifiera metoden efter resultatet. Två viktiga punkter för en lyckad analys var att använda sig av en sinigrin kalibrerings kurva och sulfatas som hade blivit renat. Resultaten från analysen visade att en glukosinolat var den dominerande i alla prover som troligen är sinalbin. Tjugotvå olika *Lepidium campestre* fröprover från tre olika länder analyserades på glukosinolat innehållet. Resultatet visade att innehållet av glukosinolater varierade mellan 180 och 360 μmol/g i alla prov förutom ett prov från Sverige som hade en halt av nästan 600 μmol/g, medelvärdet för proven från Sverige var 289 μmol/g, från Tyskland 273 μmol/g och från USA 292 μmol/g. Det fanns ingen signifikant skillnad i glukosinolat halt mellan prover från olika länder.

Nyckelord: Lepidium campestre, Fältkrassing, Glukosinolater, Sinigrin, Sulfatas, Sinalbin

Table of contents

List of tables

List of figures

Abbreviations

GSL	Glucosinolate
L. campestre	*Lepidium campestre*
(RP-)HPLC	(Reversed-Phase) High Performance Liquid Chromatography

1 Introduction

1.1 Background

The ever-growing population of the world is facing several problems with one being the food shortage. We are expected to be around 9.1 billion people by the year 2050. This amount of mouths that need to be feed is not possible with the cultivation methods used today (FAO, 2017).

Field cress (*Lepidium campestre*) is an undomesticated, native crop in Sweden and is mostly grown in the centre of the country but is still considered a weed and does not yet exist on the food market compared to some family mem bers that are already on the market, for example rapeseed (*Brassica napus*). *L. campestre* contains quite high amount of oil and is therefore one of the aspects for looking into this crop. *Lepidium* and *Brassica* come from the same family, but they do not share the same genus. Field cress can handle cold environments better than rapeseed which means that it can be sown further north in the country than rapeseed (Andersson *et al*, 1999). This could lead to more oil production and local feed for animals produced in Sweden instead of importing it, which in the long run helps the environment.

Field cress can be used as a catch crop in the cropping system due to its ability to absorb and hold nitrogen which will minimise the nitrogen leaching (Ivarson *et al*, 2016). It's a perennial crop and can work well with barley, minimizing the tillage (Börjesdotter, 2000). It has been shown that the seed yield of barley can be increased when grown together with barley (Ivarson *et al*, 2016). Field cress seeds that exist today contain high levels of linolenic acid which can be used as biofuel (Merker *et al,* 2009). However, to be used as a food or feed this has to change due to oxidative instability which will cause the product to go rancid quicker (Farhoosh *et al,* 2009). The oil also contains quite high levels of erucic acid which has been shown to have some toxicity which has been removed through breeding in rapeseed (Conor, 1999). Field cress seeds have both positive and

negative aspects when it comes to the growing and harvest aspect. The seedpods are too small for the rapeseed beetle to lay its eggs on which lowers the use of pesticides however, the seed pods cracks and falls off earlier and in an uneven rate which causes problems for the farmer when it comes to the harvest and affects the economical factor (Börjesdotter, 2000).

Glucosinolates (GSL) have been observed since the 17th century but were not characterised structurally until 1897. The structures were however, incorrect. Although this was thought to be true until 1956 when new structures were purposed (Polat, 2010). *L. campestre* contains glucosinolates which, together with an enzyme myrosinase, creates a pungent odour called mustard bomb. This is not a desirable trait to keep if field cress would be used on the food and feed market.

1.2 Project description

This study is part of a bigger project from Mistra Biotech. The vision is to contribute to make the agricultural plants healthier with higher quality and to put less strain on the environment and our farmers (Mistra Biotech Annual Report 2013). Both traditional plant breeding and gene modification techniques is used in this project. The goal is to provide the market with a perennial crop, i.e. *L. campestre,* that can survive the winter and protect the ground while being able to be used as food or feed.

1.3 Objectives

In this part of the project the objective was to develop a fast and reliable method to analyse the glucosinolate types and content in field cress seeds. The goal was also to determine the amount of glucosinolates in 22 field cress samples originating from 3 different countries.

2 Literature review

2.1 Field Cress

Today we are facing a problem with soil erosion, something that has been going on for decades. Almost a third of the world soil is already lost and the future of food production is facing a big problem (Simpson & Ogorzaly, 2001). Today there is around two million known plant species with around 300 000 of them that is actually edible and of these 300 000 only around 150 are used on the global market (Simpson & Ogorzaly, 2001). Thus, there are many crops that is not used on the food or feed market. Projects that bring the wild crops forward and use the crops natural behaviour to help in agricultural systems is therefore needed to feed our ever growing population. Today, annual crops are mostly grown and affect the soil negatively (T. S. Cox *et al*, 2002).

The use of perennial crops can help with soil erosion, however, there is a shortage of perennial crops that can grow in the nordic environment. Field cress is a perennial crop that survives in colder environment and therefore could be used further north than e.g. its relative *Brassica napus* (rapeseed). Fields cress has been shown to contain more dietary fibre and protein and less crude fat than e.g. bitter cress (*Barbarea vulgaris)*. This is interesting because both field cress and bitter cress (and rapeseed) belong to the *Brassicaceae* family which makes them somewhat comparable (Andersson *et al,* 1999). Bitter cress contains GSL and therefore has similar problems as field cress if it would be adapted to the food or feed market. The reason why the fibre content in field cress is higher is because of its thick seed coat (Merker *et al*, 2009). This could be a positive factor for human health.

The oil content in field cress is similar to the ancestor of today's rapeseed with high levels of erucic acid and linolenic acid although field cress contains even higher levels of linolenic acid (Table 1) (Sandelius, 2017). Erucic acid is not attractive when developing a healthy and stable product due to its toxicity. Linolenic acid has several health benefits though and is even deemed essential (Connor, 1999).

However, linolenic acid is prone to oxidation and can cause the product to go rancid faster than what is economically bearable (Farhoosh *et al,* 2009).

Table 1. Modified table of the oil content and the content of the fatty acids of linolenic acid and erucic acid in field cress and rapeseed oil (From Sandelius, 2017)

	Oil content (%)	Linolenic acid (%)	Erucic acid (%)
Field cress[ab]	20[a]	33-39	22-25
Native rape[c]	-	9	45
Canola oil[d]	38-44[c]	10	<0.5

[a] Nilsson *et al, 1998*
[b] Andersson *et al*. 1999
[c] Eskin *et al*. 1996
[d] Madawala *et al*. 2012

Field cress has an upright stem and branches only in the top (Ivarson *et al,* 2013). It's a diploid species that has a chromosome number of 2n=16 (Nilsson *et al*, 1998). Generally, the plant can reach a height of around 60 cm and has white flowers. Each seedpod contains only two seeds which are very small compared to rape seeds. Because of its small size the rapeseed beetle can not lay its eggs on the flower buds (Börjesdotter, 2000).

Field cress has been sown together with barley and it has been shown that sowing these two crops together can have a positive effect on both the barley yield and leaching of nutrients (Merker *et al,* 2009). There are certain traits of field cress which has to be adjusted before it is suitable as either food or feed, for example the low oil content, the fatty acid profile, the high glucosinolate content (Ivarson *et al,* 2016) and that the seedpods cracks and falls in an uneven rate on the field, which causes an economical loss (Börjesdotter, 2000).

Figure 1. Field cress (Wikipedia).

2.1.1 Environmental factors

As mentioned above, the use of perennial crops can have a positive effect on the environmental side of farming. When the fields are spared the process of tillage, it affects the nitrogen leaching in a positive way. It also reduces the risk of soil erosion and can probably make the carbon content in the ground to increase. The lesser use of heavy machinery on the field lower the stress on the ground and therefore the soil compaction is lessened. Perennial crops can create rhizome systems underground where bundles of roots are created over time (Lewandowski e*t al,* 2003). These bundles can recycle their own nutrients and therefore not have the same need for extra nutrients being added which saves the farmer both time and money. When talking about perennial grasses it has been shown to have a positive effect on the fauna because of the longer time undisturbed by humans and the late harvest (Lewandowski *et al,* 2003).

2.1.2 Economic factors

Since field cress is a perennial crop, the farmer saves money on both time and fuel, but there have also been studies done on how the seed yield is affected by this. Barley that is sown together with field cress has been shown to have an increased seed yield which in turn gives more money back to the farmer (Merker *et al,* 2009). The seed yield in field cress has not been shown to increase by being grown together with barley, however. Today neither the oil nor the meal from field cress is attractive

for food or feed use. The oil contains, however, a lot of linolenic acid which could be used in an industrial purpose as a biofuel for example (Merker *et al*, 2009)

2.1.3 Canola oil development

Rapeseed has been used for centuries and is still used today in high quantities all over the world. However, the seeds that are used today are not genetically the same as its ancestor. Many breeding programs have worked to remove or lower levels of unwanted compounds. The level of erucic acid was lowered through a breeding program in Canada in the 1960, which was implemented in *Brassica napus, Brassica rapa and Brassica juncea.* Another problem was the high level of GSL in the seed meal. Because of GSL pungent smell and taste it caused the animals not to eat it, GSL also has some negative health aspects. The lower content of GSL was incorporated into *Brassica napus and Brassica rapa.* When a rapeseed is successfully breed with less erucic acid (less than 2%) and less GSL (less than 30 μM/g) it can be considered as canola oil (Gunstone, 2004). The oil composition was also lowered due to the oxidative properties of the oil, in table 1 its shown how the rape seed oil has changed.

2.2 Glucosinolates

Glucosinolates, β-thioglucoside-N-hydroxysulfates or mustard oil (Figure 2), is the same product under different names (Fahey *et al,* 2000).

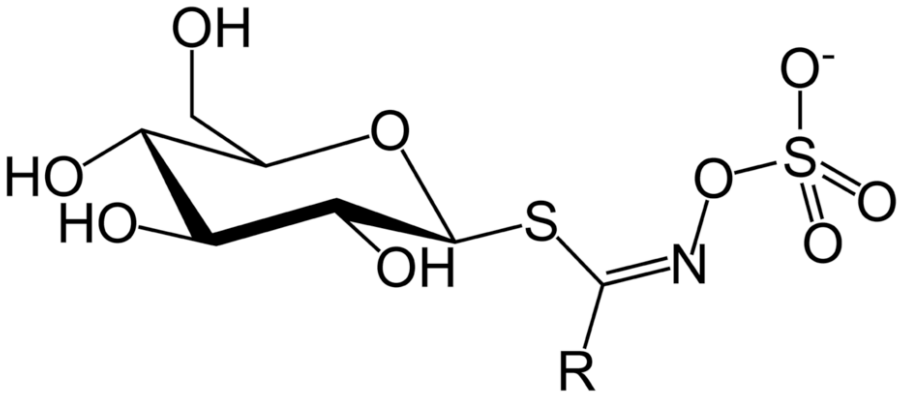

Figure 2. The chemical structure of a glucosinolate. The R-group will differ between the different glucosinolates (Wikipedia).

Today we know about 130 different GSL. These are characterised by their structure, but they are also separated into classes depending on their amino acid. For example, indole GSL is synthesized from tryptophan and aromatic GSL is synthesized from phenylalanine (Grosser & van Dam, 2017). When separated by their structure there is around 10 classes, with some being more explored than others. These are aliphatic, ω-methylthioalkyl, aromatic and indole GSL (Fahey *et al*, 2000). There is normally around 2 mmol/ kg of fresh weight of GSL in plants from the *Brassicaceae* family (Coultate, 2014).

GSL is a secondary metabolite in plants that coexist with the enzyme myrosinase β-thioglucosidase. GSL is chemically stable before coming in contact with myrosinase, which happens when the plant is damaged (Song & Thornalley, 2007). When the tissue is damaged in the plant and glucosinolate reacts with myrosinase there are some new products that forms, usually nitrile, isothiocyanate and thiocyanate (Figure 5) (Hanschen *et al*, 2015). Epithionitriles can be formed from unsaturated aliphatic GLS due to epithiospecifier protein that exist in some *Brassica* plants (Hanschen *et al*, 2015).

GSL is stored in the vacuoles in the plant cells which separates them from the enzyme that is present in the cytoplasm (Grosser & van Dam, 2017). When they react with each other a pungent smell, also called a mustard bomb, will be created to protect the plant from herbivores or pests when there is tissue damage (Grosser & van Dam, 2017). The enzyme reaction catalyses on the carbon-sulfur bonds which in turn frees thiohydroxamate-O-sulfates (Coultate, 2014). A method to denature myrosinase and avoid the GSL to be damaged is to boil the samples, which will inhibit the conversion of the GSL to isothiocyantes to a certain point (Song & Thornalley, 2007). If *Brassica* vegetables is consumed raw or treated by for example steaming, stir-frying or microwaving there is still a lot of unconverted GSL. These GSL can be degraded in the gut by bacteria (Song & Thornalley, 2007). Most frozen foods that are pre-chopped are usually blanched or steamed before frozen, which inactivates the myrosinase and cause minimal degradation of GSL when thawed. But if the vegetable is frozen without a pre-treatment there is a risk of freeze-thaw fracturing during thawing which causes the GSL to be degraded (Song & Thornalley, 2007).

Certain products of GSL can have an effect on human and animal health, some even being anti-nutritional and toxic (Mellon *et a*l, 2002). The forms that are most associated with toxicity are β-hydroxylalkenyl and indole GSL (Shapiro, 2001).

Plants that contain GSL need to be breed in a way so the amount of GSL does not affect the food or feed in a negative way but at the same time the amount needs to be high enough to protect the plants against predators (Coultate, 2014). The change from high level rapeseed plants to low level rapeseed has shown different

effects on different pests. The flea beetle seems to be unaffected by the lower GSL content however, diamondback moth is affected because GSL simulates their oviposition which in the end will be negative to the plants (Bodnaryk, 1997).
There are several GSLs detected in several different plants and vegetables, Some of the more common is glucoiberin in broccoli seed extract (Troyer *et al*, 2001) and progoitrin and epiprogoitrin among others in rapeseed (Millán *et al*, 2009). Most plants and vegetables have several GSL which exist in high concentrations, however, some plants have only one really dominant GSL for example Land cress (*Barbarea verna*) which is dominated by gluconasturiin and wallflower (*Cheiranthus cheiri*) which has glucoiberin as its dominant GSL (Matthäus *et al*, 2000).

In this study, there has been a focus on sinalbin because, according to earlier studies (Andersson *et al,* 1999) this is the dominant GSL in *L. campestre*. The reason for also focusing on sinigrin is because it was used as an internal standard during the present experiment. This was made possible because of its commercial availability.

2.2.1 Sinigrin

Sinigrin (2-propenyl) (Figure 3) is a GSL that can be found in *Brassicaceae*- and *Capparaceae* families and is classified (by chemical structure) as an olefin, which is the class of straight and branched chain compounds (Fahey *et al*, 2001). Sinigrin was first discovered from black mustard seeds *(Brassica nigra)* in the 1830's. The amount of sinigrin reflects the "hotness" (not an official scale) of the product (Tsao *et al*, 2002). Sinigrin has been perceived to not be pleasant when eaten because of its pungency. It is also the dominant GSL in many plants and vegetables which means that it can't be masked by more pleasant GSL (D'Antuono *et al,* 2009). Sinigrin could become severely toxic if hydrolysis happens, which would result in mustard gas being produced. This compound however, is non-existing in rapeseed plant because aliphatic GSL does not exist in rapeseed (Wretblad & Dixelius, 2000). Sinigrin has been shown to be an effective pesticide *in vitro* against *Heterodera schachii,* a roundworm that affects many plants across the world. In an experiment sinigrin (0.5%) was given to second-stage *H. schachii* and 100% was dead after 24 hours being the highest mortality rate of the seven GSL used in the experiment (Lazzeri *et al,* 1993). In another study, it was shown that sinigrin helps protect *Brassica juncea* from bertha armyworm because of the high levels in the leaves (Bodnaryk, 1997).

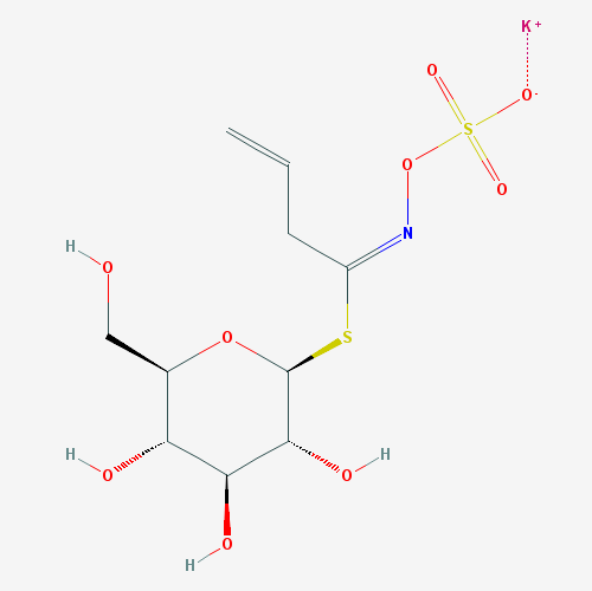

Figure 3. The chemical structure of sinigrin (pubchem).

2.2.2 Sinalbin

Sinalbin (4-hydroxybenzy) (Figure 4) can be found in *Brassicaceae-, Capparaceae-, Moringaceae-, Phytolaccaceae-, Resedaceae-, Salvadoraceae- and Tropaeolacea*e families, and is classified as an aromatic GSL (Fahey *et al,* 2001). Sinalbin, just as sinigrin, was discovered in the 1830's but in white mustard seeds (*Sinapis alba)*. In earlier experiments sinalbin was found to be the main glucosinolate in field cress (Andersson *et al,* 1999). Sinalbin has a dominant herbaceous taste and compared to sinigrin the taste is not as strong in flavour (D'Antuono *et al,* 2009). Sinalbin has been tested *in vitro* to see its nematocidal effect on *H. schachii* and it had an effect, however, it was not as good as sinigrin. Sinalbin had a mortality effect of less than 10% after 96 hours. This is much lower effect and takes longer time than for sinigrin (Lazzeri *et al*, 1993).

When sinalbin is degraded by myrosinase the main component (in mustard paste) was 4-(hydroxymethy) phenol. 4-(isothiocyanatomethyl) phenol that has been perceived to be the reason for the pungent smell, was not detected in mustard paste, which means that in mustard paste this is not the cause for the pungent smell or taste (Paunović *et al*, 2012). In a study comparing high- and low levels of GSL it was shown that sinalbin can help with protection while the plant is young, but is not that effective when the plant is grown older. Sinalbin levels in *S. alba* is very high in the present crops and if the sinalbin content would be lowered in the seeds the

plant would be at risk for seed damage from the lygus bug who is affected negatively by sinalbin (Bodnaryk, 1997).

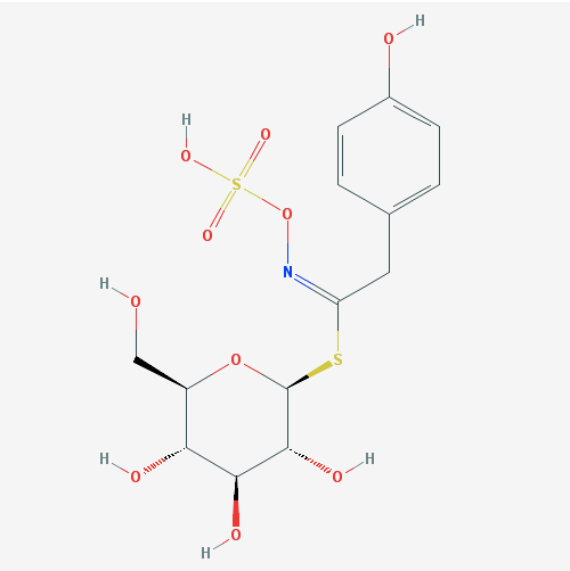

Figure 4. The chemical structure of sinalbin (pubchem).

2.2.3 Effects of GSL

Different GSL is perceived differently. Whole GSL is often perceived to be bitter while isothiocyanates can give a burning sensation and other products can taste rotten because of the sulphur. Although the bitter taste is a natural protection for the plant, humans seems to find it as a positive factor in the sensory aspect (D'Antuono *et al*, 2009).

Today there is not shown to be a toxicity risk for humans who consumes *Brassica* vegetables in a "normal" fashion. For example: if a person weighing 70 kg eats 200 g of uncooked savoy cabbage there will probably not be any acute toxic reaction. However, there is more research needed in the area because some toxicity has been shown in rats and mice (Hanschen *et al*, 2015).

Some studies have been done on using isothiocyanates on human cancer cells which has shown little damage to healthy tissue and toxicity towards liver cancer cells. Epithionitiles seems to have cancer prevention properties but there are also studies showing toxicity to the liver and kidney in mammals (Hanschen *et al*, 2015). When rapeseed meal of the presscake after rapeseed oil extraction is used there is a risk of toxicity due to the isothiocyanates having a hydroxyl group placed in the β-position. This could create compounds that are collectively called goitrins (Coultate, 2014). Goitrins can affect the thyroid gland by disturbing the iodine uptake, which

in turn cause enlargement of the gland and cause deficiency of thyroxine (Coultate, 2014).

GSL and the degradation products could be called Janus (having two faces) because they could be both carcinogenic and anticarcinogenic depending on the condition. We do not know the dosage line between these two as the testing is primary still on animal testing stage (Polat, 2010).

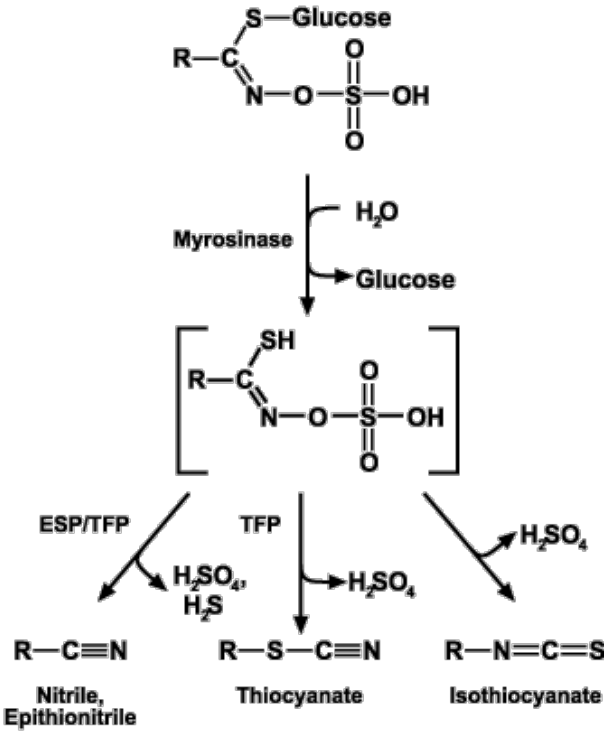

Figure 5. Glucosinolate reaction with myrosinase and the degradation products. (Google).

3 Material and Method

3.1 General aspects

The method of glucosinolate analysis was developed according to Grosser & van Dam (2017) with modifications as described below.

Because myrosinase starts to degrade GSL as soon as the sample is milled the samples were boiled in methanol as soon as possible, this was also done to extract the GSL. Purification of GSL was performed using Sephadex A-25 columns. The negative sulfate groups on GSL will stick to the columns and are removed from the eluted compound by using a sulfatase. The desulfoglucosinolates are released and washed out with milliQ water and collected. The GSL is separated in a reversed phase- HPLC column and was detected around 229 nm.

Some parts of the method from Grosser and van Dam was never included to the method from the beginning. These steps were: freeze drying of the sample flour, ultrasonic water bath during extraction, freeze drying the samples before HPLC. The steps were removed because they were overlooked or replaced by another method.

3.1.1 Seeds

The seeds used for method development were two different undefined *Lepidium campestre* samples, here called Lepidium (A) and Lepidium (B). The 22 *Lepidium campestre* samples analysed for content of GSL were all grown in Sweden. However, they originated from three different countries (Germany, USA and Sweden) according to Table 1.

3.1.2 Reversed phase high performance liquid chromatography

The column used in the RP-HPLC was a reversed-phase C18 named HP ODS Hypersil. Measurements of the column was 125×4 mm with a 5 μm diameter filter.

The HPLC program had a flow rate of 0.750 ml/min and was run for 20 min. This was accompanied by a temperature of 40°C in the column oven and a UV lamp with a wavelength of 229 nm. For the gradients water (solvent A) and acetonitrile (solvent B) was used. The program was: 0% B (0 min), 10% B (10 min), 35% B (15 min), 0% B (20 min) and a post time for 5 minutes with B. GSL was detected at 6-7 minutes.

3.1.3 Dry matter

When a new flour samples was prepared for analysis 2 × 100 mg of the sample was weight for determination of dry matter content. Samples were weighed in tin forms and put in an oven at 105 ºC for 16 hours, and then re-weighted.

3.1.4 Preparation of DEAE Sephadex A-25 columns

The columns were prepared a few days before use so that the gel had time to set. Sephadex A-25 powder (2 g) was mixed with 20 ml 0.5 M acetate buffer at pH 5. The suspension was filtered through a Munktell filter paper and the gel was resuspended with 20 ml 0.5 M acetate buffer at pH 5. Then the gel was filtered again and washed with water. The gel was collected and diluted two times with 0.02 M acetate buffer at pH 5. The gel was then used to make the columns.

Plastic pipettes (1 ml) were mounted vertically and a small amount of glass wool was put in the bottom of the tip. Waste tubes were mounted beneath the tips and 1 ml of the Sephadex gel (well stirred) was added. The liquid was left to drain. The tubes were then washed with 1 ml milliQ water before covering with parafilm and put in a refrigerator.

3.1.5 Internal standard

An internal sinigrin standard was used which was made by dissolving 0.02 g of sinigrin monohydrate in 10 ml of milliQ water making the concentration around 6 mM.

3.1.6 Sulfatase

The sulfatase used was from *Helix pomatia* (Roman snail) with an activity of ≥2,000 units/mL (Sigma-Aldrich). The sulfatase was added directly to the sephadex column, without any purification.

3.1.7 Milling

The seeds *L. campestre* were milled using a coffee grinder (Braun), not letting the grinder get too hot during the process. The grinding program was: 5 seconds on level 1, 15 seconds on level 2 and 40 seconds on level 3. After one minute the machine was turned off level by level instead of shutting it down directly. During the setup of the method 50 g of Lepidium seed samples was milled at the same time and kept in a refrigerator for the duration of the experiment.

3.1.8 Extraction

For the extraction of GSL, 70% MeOH was heated to 80-100 ºC and added to 250 mg of the flour in 15 ml Falcon tubes. This was done in duplicates. Hot MeOH was added to the 3 ml mark on the Falcon tube. The sinigrin internal standard was added (50 µl) to the mixture and the tubes were then mixed and immediately put into a boiling water bath for 15 minutes. The mixtures were vortexed halfway through the boiling time. The tubes were cooled to room temperature before centrifugation at 4000 rpm for 5 minutes. The supernatant was used directly or put in the freezer at -20 ºC until desulforylation.

3.1.9 Purification and desulforylation

The DEAE Sephadex columns were mounted vertically above waste tubes. All of the extract was carefully added to the columns and left to drain. The columns were then washed with 2 × 1 ml milliQ water and left to drain in between the washings. Thereafter the columns were washed with 2 × 0.5 ml 0.02 M acetate buffer, pH 5.

New clean collection tubes were mounted under the columns. Then 75 µl sulfatase was added to the columns and left overnight covered with parafilm in room temperature. The desulfo-GSL were eluted with 3 × 0.3 ml milliQ water and the eluted samples were collected. The samples were then evaporated as much as possible by adding the samples to a small centrifuge with a heating lamp above, the samples were run for three hours before this was stopped, some water was still left.

The semi-evaporated samples (50 µl) was transferred to a HPLC vial before analysis with HPLC.

3.2 Method development

During the first run of the method it was made clear that the internal standard would not work due to the low concentration of sinigrin compared to GSL content in the samples, dilutions was made on the raw samples to see if this would be enough to use the internal standard. This, however, did not work. The internal standard was therefore replaced with a sinigrin standard curve as an external standard.
The second change was the sulfatase. The sulfatase was purified because of its side activities this could be one reason for the big differences in GSL concentration between the replicas that was observed. The samples were diluted 10 times before the HPLC due to the high peaks on the chromatogram. During the modified method, it was decided to study the GSL during storage of *Lepidium* flour when kept in a refrigerator. The variation in the method within days and between days was also studied in two different trials.

3.2.1 Sinigrin standard curve

A stock solution of sinigrin monohydrate was made with a concentration of 2.22 mM in 10 ml milliQ water. Five reference samples between 50-700 µM were prepared by dilution of the stock solution. The reference samples were frozen in 1 ml Eppendorf tubes for future use. For the last batch of samples a new sinigrin stock solution with a concentration of 2.07 mM was made.

3.2.2 Sulfatase solution

For the modified method, sulfatase was purified by mixing the sulfatase with 6 ml milliQ water and 6 ml ethanol. The mixture was then centrifuged at 2650 × g for 20 minutes. The supernatant was transferred to a beaker and 18 ml of ethanol was added. The mixture was centrifuged again at 1030 × g for 15 minutes. The supernatant was discarded and the pellet was dissolved in 5 ml of milliQ water. The mixture was transferred to 1 ml Eppendorf tubes and frozen for future use (Grosser & van Dam 2017).

3.3 The between days variation of GSL content

For determination of variation between days for the analysis, fresh samples were milled and put in the refrigerator before use, or used directly, the samples were analysed on day 0, day 6, day 7 and day 13. The samples (250 mg) were extracted with hot methanol in duplicates as described above.

The extract (3 ml) was added to the DEAE sephadex columns as described above, and after washing with milliQ water and acetate buffer, 75 µl of the **purified** sulfatase was added. The columns were washed with 3 × 0.3 ml milliQ water and the eluted samples were collected the day after. The exact eluted volume was measured and later used to calculate the content of GSL. The samples were not evaporated. The same procedure was also applied to the sinigrin references. The samples were diluted 10 times with milliQ water, and 50 µl was transferred to a HPLC vial before analysis with HPLC. The HPLC program was set as above (3.1.2).

3.4 Analysis of samples from different origin

The amount of flour was used was decreased from 250 mg to 100 mg and instead of adding methanol to the 3 ml mark exactly 3 ml of methanol was added. Only 1 ml of the raw sample was added to the columns because of concerns of overloading the columns.

The 22 *Lepidium* samples from different countries analysed are given in Table 2. All were frozen directly after grinding.

Table 2. Seed samples of *Lepidium campestre* from three different countries

Sample	**Country**
Ljungarn	Sweden
Mörbylånga 109	Sweden
Mörbylånga 110	Sweden
Gävle-3	Sweden
Arrie	Sweden
Spjutstorp	Sweden
Malmö	Sweden
Huddinge	Sweden
Grönhögen	Sweden
Lc 251	USA
Lc 251-2	USA
Pi 650260	USA
Lc 251-6-53	USA
Pi 633248	USA
Lc 251-3B	USA
92-9-98	Germany
Lep 92-3	Germany
Lep 92-2	Germany
Lep 92-9	Germany
92-9-173	Germany
Lep 122	Germany
92-6	Germany

3.4.1 Extraction and analysis

For the analysis of the 22 different *Lepidium* samples from different countries (Table 2) samples (100 mg) were milled and immediately transferred to the freezer. The samples were taken out and thawed when needed. The grinding program was the same as in the beginning of method development. The grounded flours were extracted with exactly 3 ml methanol in duplicates.

Only 1 ml of the extract was added to the DEAE-Sephadex columns to avoid over loading and after washing as before 75 µl of the purified sulfatase was added. The columns were treated as descried in 3.3 however, the sephadex columns was

washed with 6 × 0.3 ml milliQ water (double the amount). The samples were therefor diluted 5 times before analysis with HPLC.

3.5 Statistical analysis

For the results from the 22 Lepidium samples from 3 different countries a one-way ANOVA was done to analyse if there was a statistical difference between the different countries. This was performed with Minitab.

4 Results and discussion

4.1 General aspect

Figure 6 shows a typical chromatogram of a GSL extraction from *L. campestre*. There was one large peak and several smaller ones. It was decided to focus on the large main peak (Figure 6).

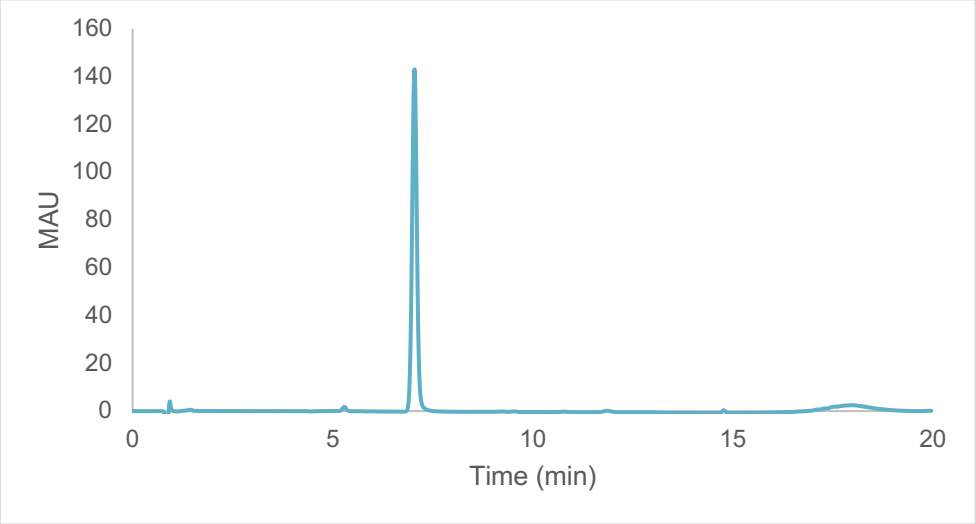

Figure 6. Chromatogram of Lepidium sample after treatment with purified sulfatase. The large peak potentially being sinalbin and the second being undefined.

The spectra from the large peak (Figure 7) matched the spectra reported by Grosser & van Dam (2017). They had identified it as sinalbin. This peak was always present in the samples. This can not be guaranteed though without further testing however,

earlier results have shown that sinalbin is the dominant GSL in field cress (Andersson *et al,* 1999). One problem in the beginning was the height of the peak which

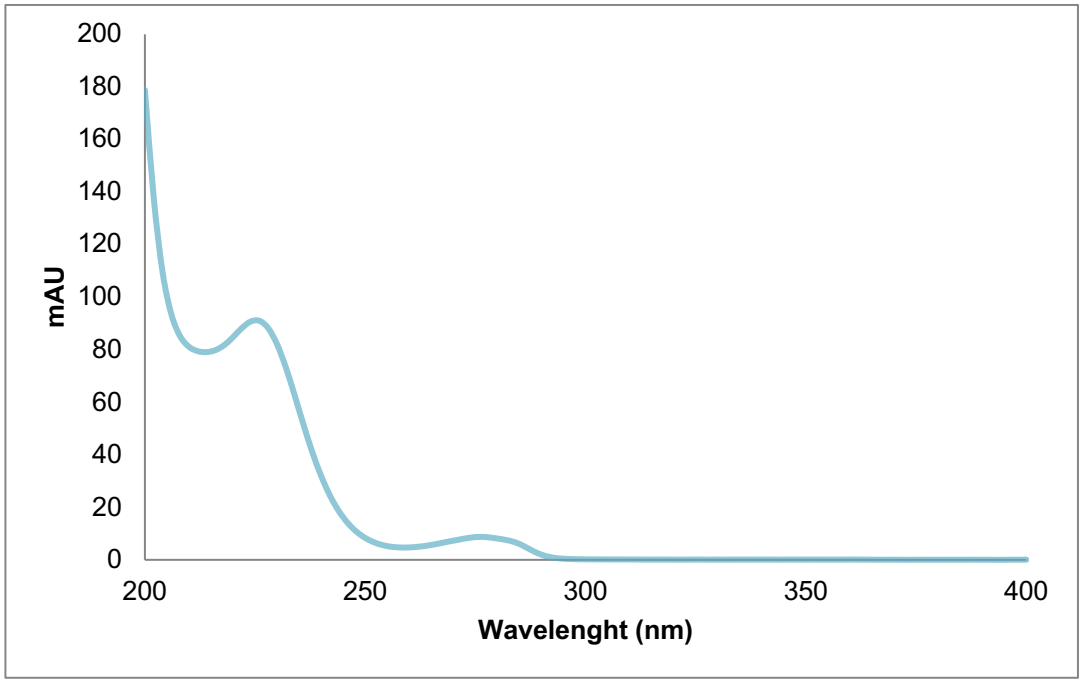

Figure 7. Spectra of sinalbin peak

was too high compared to the internal standard. Therefore, the same samples were diluted 10 and 100 times to try to match the internal standard. This gave better results but replicates varied too much for the result to be reliable. The decision to use sinigrin at all as a standard is because it is commercially available while sinalbin is not.

When analysing the spectra of the larger more defined peak in the chromatogram it shows a curve matching the one in Grosser & van Dam (2017) as shown in Figure 7. This can therefore with most certainty be sinalbin. The second peak that was less defined had a spectrum that was harder to analyse due to the fact that is does not look the same in between samples, similar but not the same. This peak was so unstable that no suggestion was made and will not be made without further testing.

4.2 Method development

In the modified method, the internal standard was replaced with an external sinigrin standard curve instead. This was done due to the low peak of sinigrin compared to the sinalbin peak. This was combined with diluted samples and use of purified sulfatase which gave more reliable results (Figure 6). The use of unpurified sulfatase affected the result negatively due to its side activity which causes many peaks to show up on the chromatogram (Grosser & van Dam 2017). When used on the sephadex columns with the raw extract several products were formed and gave several peaks in the HPLC chromatogram. Therefore, it was decided to purify the sulfatase by treating it in ethanol (see method). This showed to give better results, with sharper peaks and less small peaks that clustered together (Figure 6). Due to the charge on the columns the negatively charged sulfate groups will stay in the column during the washout (Grosser & van Dam 2017).

In the beginning, 250 mg sample were used and 3 ml of the raw extract was added to the column. Later there was thoughts of overloading the column with the raw extract. Therefore, a smaller amount of samples was tested (100 mg) and only 1 ml of the raw extract was added to the column. There were no obvious differences in the results, and therefore the smaller amount was used for further analysis.

4.3 The within and between days variation of GSL

4.3.1 Trial 1

It was found that the content of sinalbin became lower over time in the flour samples, which were stored in the refrigerator (Figure 8 & 9). The content was decreased from 167 to 70 µmol/g for *Lepidium* (A) and from 183 to 100 µmol/g in *Lepidium* (B) over a 13 day period.

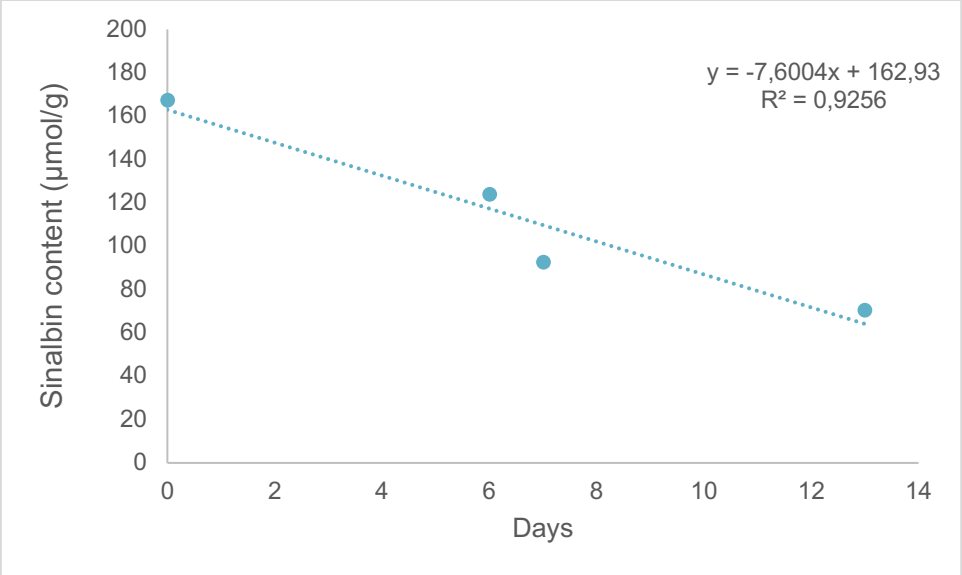

Figure 8: Sinalbin content in flour of *Lepidium (A)* after different days of storage in a refrigerator mean value of 4-5 replicates.

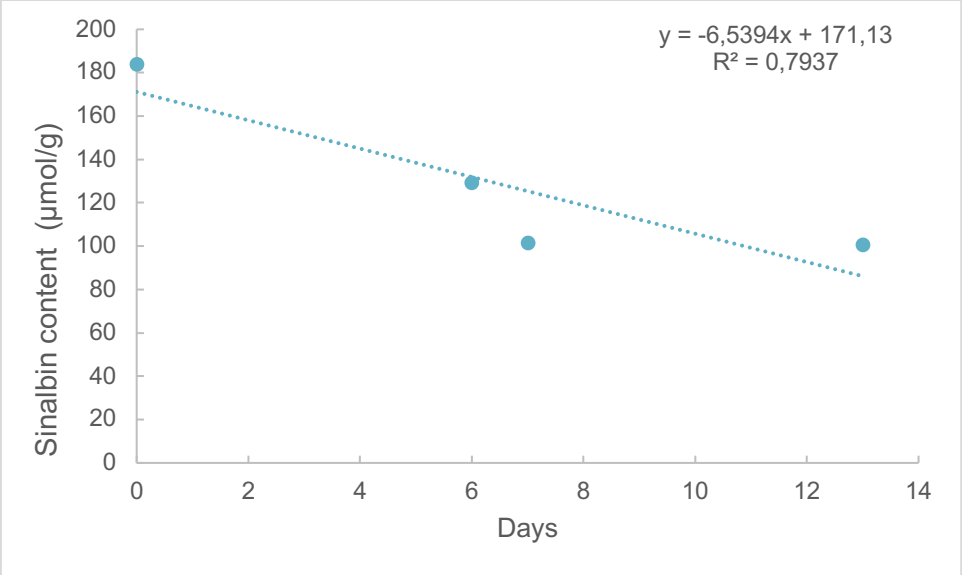

Figure 9: Sinalbin content in flour of *Lepidium (B)* after different days of storage in a refrigerator mean value of 4-5 replicates.

The variation within days was determined in this experiment and the coefficient of variation was 10 to 18% in *Lepidium* (A) and 4 to 18 % in *Lepidium* (B) (Table 3).

Table 3. Content of sinalbin in 4 or 5 replicates of *Lepidium (A)* and *Lepidium (B)* at different days of storage in a refrigerator (µmol/g), CV (%) within day of analysis.

	Day 0		**Day 6**		**Day 7**		**Day 13**	
Replicas	A	B	A	B	A	B	A	B
1	267[1]	182	144	140	74	88	86	87
2	179	171	110	161	106	115	56	105
3	175	190	125	137	75	95	74	124
4	146	189	114	105	96	116	64	83
5			124	100	109	90		
Mean	167	183	123	129	92	101	70	100
CV %	10	4	10	19	17	13	18	18

1. This number is not used in the calculation due to the fact that it does not fit and has an error.

4.3.2 Trial 2

As the content of the sinalbin decreased in the refrigerator stored samples it was not possible to calculate the between days variation. Therefor new samples were milled to analyse the variation of results between days of fresh samples that were milled on different days. The variation between days was calculated from trial 1 day 0 and trial 2 day 0 and the difference was 26% for *Lepidium (A)* and 17% for *Lepidium (B)* (Table 4). Mean content of sinalbin in 4 replicates of Lepidium (A) was 167 µmol/g in trial 1 and 128 µmol/g in trial 2. Corresponding values for *Lepidium (B)* were 183 µmol/g for trial 1 and 155 µmol/g for trial 2.

Table 4. Content of sinalbin in 4 replicates of *Lepidium (A)* and *Lepidium (B)*. Difference (%) for each sample is given in fresh samples in trial 1 (day 0) is compared to fresh samples from trial 2 (day 0).

	Trial 1 (day 0)	**Trial 2 (day 0)**	**Trial 1 (day 0)**	**Trial 2 (day 0)**
	A	A	B	B
1	267[2]	156	182	172
2	179	113	171	151
3	175	99	190	14[2]
4	146	144	189	144
Mean	167	128	183	155
	Diff. 26%[1]		**Diff. 17%[1]**	

1 Difference (%) was calculated as difference between trial 1 and trail 2 divided by the mean value of the of the mean of trial 1 and 2 This number is not used in the calculation due to the fact that it does not fit and has an error.

4.4 Analysis of samples from different origin

To try to reduce the variation within days of analysis, we tried to use less amount of sample to avoid overloading. Therefore, 100 mg of fresh samples were weight in instead of 250 mg. However, this did not really make a big difference. The contents of sinalbin were generally around 100-175 µmol/g in *Lepidium* (A) and *Lepidium* (B). The amount of water that was used in the washout process from the column before the HPLC analysis was 3 × 0.3 ml in the beginning of method development. There was a discussion if this volume was enough to washout the desulfoglucosinolates. Therefore, the washout volume was doubled and the dilution before HPLC analysis was halved in further analysis.
All samples from Sweden except one varied between 190 and 360 µmol/g. The one sample that differed had a content of almost 600 µmol/g. This was about 67-21% higher than the other Swedish samples. Samples from Germany had a smaller variation of 200- 320 µmol/g and lastly samples from USA had a content between 170-370 µmol/g which was the largest variation (Figure 10). Samples from Sweden had a mean value of 289 µmol/g (including all the samples), from Germany 273 µmol/g and from USA 292 µmol/g (Figure 10). To calculate the amount of sinalbin in the samples, the equation from the external sinigrin calibration was used assuming similar detector responses.

The amount of sinalbin in all samples is shown in Figure 11. When comparing the results between samples from Sweden, USA and Germany there was no significant difference with a p- value of 0.909.

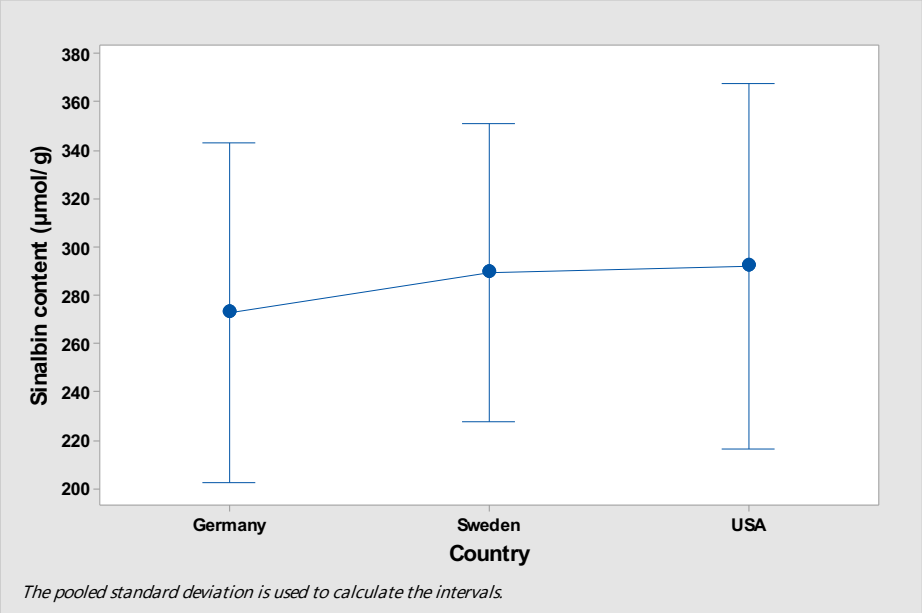

Figure 10. Interval Plot of Sinalbin vs Country ANOVA (95% CI for the Mean).

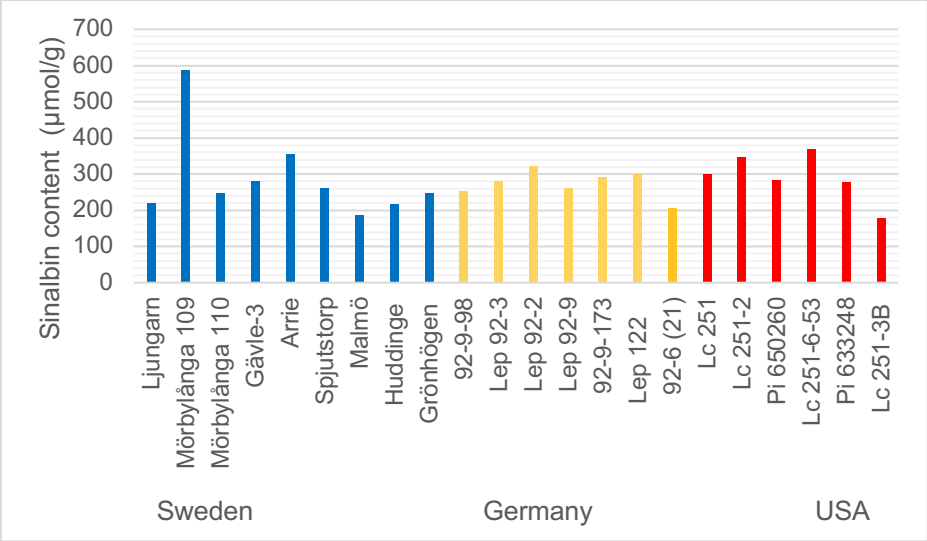

Figure 11: Glucosinolate content in Lepidium samples from Sweden (left), Germany (middle) and USA (right).

4.4.1 Re-analysis

There were some samples that on the first try did not give a satisfying result for the duplicates so a re-analysis of seven samples were performed. In the new results the mean value was higher than in the first run for the majority of the samples. The duplicates were more even and the result therefore, more reliable, and included in the results.

The samples that were re-analysed, was treated with a new mix of both sinigrin and sulfatase which was not optimal because it was not the same as in the other experiments. Something that was interesting was that the new sulfatase seemed to be more effective than the old batch. The chromatograms were cleaner and sinalbin peak appeared consistently earlier than the old samples. Even though the time difference is not much it is noticeable when comparing the chromatograms.

This might be due to the new sulfatase. The first batch of sulfatase was made in the same way but in a higher quantity. This should not affect the quality of the sulfatase but when working on the first batch a human error might have occurred. The new sulfatase was never frozen either, which in the long run might affect the sample but not likely.

4.5 Original method steps

4.5.1 Ultrasonic bath

Ultrasonic bath was used in the original method by Grosser & van Dam (2017) in between the hot water bath and the centrifugation but was not used during this study. Using an ultrasonic bath could have increased the extraction for GSL from the flour samples. This was confirmed in a study by Albu *et al* (2004).

4.5.2 Evaporation

In the beginning of method development, the water was evaporated from the samples before the HPLC analysis. This was done by using a centrifuge. However, the internal heat system was broken so a heating lamp was used on the outside of the machine. This took a very long time and as a more critical point: the samples were exposed to heat during a long period of time. The samples did not however become completely dry. Therefore, it was decided to compare some evaporated samples suspended in water with some that were not evaporated (from the same batch). There

was no obvious difference between the samples. Consequently, the evaporation step was removed. To get correct concentrations of glucosinolates, the eluted volumes were instead measured exactly before HPLC analysis.

4.6 Suggestions for future development of the method

4.6.1 Columns

The sephadex columns that was used during the experiment was handmade which gives a risk for human error. They were carefully checked for abnormality's and made sure that they were as equal as possible. Something that could be a big factor in the gel quality was that during stirring of the gel, a tool was sometimes used. This could damage the gel. Therefore, a swirling motion should be used as much as possible. A solution on this problem could be to buy columns that are professionally made.

4.6.2 Boiling for extraction

Something that needs to be taken into consideration is that by boiling the samples for extraction of GSL, not all of the myrosinase is inactivated. This is apparently a bigger problem if the moisture content is lower than 8% (Herzallah *et al,* 2012). In our samples, the moisture content was between 7-9% so this is something to take into consideration when grinding the samples.

4.6.3 Extraction of seeds

The seeds in this study were milled without any pre-treatment. However, in a study performed by Mellon *et al* (2002) the seeds were dried at 100°C for 24 hours before milling. This was done to inactivate myrosinase. Since we have observed myrosinase activity in the first samples and know that they are still active in a refrigerator this might be an option for inactivation of the enzyme. Another solution to this could be to keep the samples in a freezer and grind the samples in smaller batches.

The methanol was used for extraction and to stop the reaction between glucosinolate and myrosinase. However, this post a problem later in the experiment. Because the seeds are grounded in the preparation step there might be a reaction between the glucosinolates and myrosinase. To stop this reaction the seeds could be

milled in liquid nitrogen to keep the samples frozen until the methanol is added (Mellon *et al,* 2002). Suggestion from Herzallah *et al* (2012) is to use water during the extraction to minimize the risk of residues of methanol to obscure the results. However, this seems not to be a problem during our study. Another solution would be to grind the seeds in the hot methanol directly. This posts some practical difficulty by itself but is however not impossible.

4.6.4 Calibration curve

The calibration curve worked generally well throughout the study. However, there were some problems in the later runs with peaks not showing in the HPLC. This could be because of the period in the freezer, but this is not likely since sinigrin is stable at 4°C and below (Tsao *et al,* 2002). Something that could be done in the future is to run the sinigrin samples several times and calculate an average to ensure a more accurate calibration curve, which was done by Tsao *et al* (2000).

4.6.5 Sulfatase

In the form that the sulfatase was added on the sephadex columns and left overnight there is the question if the sulfatase really had time to interact with the whole column and all of the sample. This could not be guaranteed or controlled. A way to make this in a controlled way would be to add the sulfatase to the raw extract before adding it to the column but doing it before the washing would not work since the sulfate binds the glucosinolates to the column. One suggestion could be to mix the enzyme solution with the column material after the washing step to ensure a complete enzyme action before eluting the sample.

4.7 Conclusion

In this study, the focus was on developing a fast method to analyse glucosinolates in *Lepidium campestre* in a reliable way. The method is not yet reliable, since the variation in GSL content between replicates is too high. However, the method has come far and method development can be continued from this work and with the suggested changes the method could become successful. The results showed that sinalbin is the dominant glucosinolate in *Lepidium campestre,* and that the content was similar in 22 different samples from 3 different countries.

5 References

5.1 Literature

- Albu, S., Joyce, E., Paniwnyk, L., Lorimer, J.P., and Mason, T. J. ”Potential for the use of ultrasound in the extraction of antioxidants from Rosmarinus officinalis for the food and pharmaceutical industry”. *Ultrasonics Sonochemistry*, 4th Conference on the Applications of Power Ultrasound in Physical and Chemical Processing, 11, 3 (2004): 261–65.
- Andersson, A.M., Merker, A., Nilsson, P., Sørensen, H., and Åman, P. ”Chemical composition of the potential new oilseed crops Barbarea Vulgaris, Barbarea verna and Lepidium campestre”. *Journal of the Science of Food and Agriculture* 79, 2 (1999): 179–86.
- Bodnaryk, R. P. ”Will low-glucosinolate cultivars of the mustards Brassica juncea and Sinapis alba be vulnerable to insect pests?” *Canadian Journal of Plant Science* 77, 2 (1997): 283–87.
- Börjesdotter, D. Oljehaltiga fånggrödor -vårgyllen, sommargyllen och fältkrassing. *FAKTA Jordbruk* [online], 2000(9). Available from: https://www.slu.se/globalassets/ew/ew-centrala/forskn/popvet-dok/faktajordbruk/pdf00/jo00-09.pdf [Accessed 2017-09-15].
- Connor, William E. ”α-Linolenic acid in health and disease”. *The American Journal of Clinical Nutrition* 69, 5 (1999): 827–28.
- Coultate, T. (2014) *FOOD The chemistry of its components*. Vol. 5. RSCPublishing.
- Cox, T.S., Bender, M., Picone, C., Van Tassel, D. L., Holland, J. B., Brummer, E. C., Zoeller, B. E., Paterson A. H., and Jackson, W. Breeding perennial grain crops (part 1 of 3). *Critical Reviews in Plant Sciences, 21(2),* (2002): *59-91*
- D’Antuono, L.F., Elementi, S., and Neri, R. ”Exploring new potential health-promoting vegetables: Glucosinolates and sensory attributes of rocket salads and related diplotaxis and eruca species”. *Journal of the Science of Food and Agriculture* 89, 4 (2009): 713–22.
- De Villena, F.A., Fritz, V.A., Cohen, J.D., and Hutchison, W.D. ”Changes in gluconasturtiin concentration in chinese cabbage with increasing cabbage looper density”. *HortScience* 42, 6 (2007): 1337–40.
- Eskin, M.N.A., McDonald, B.E., Przybylski, R., Malcolmson, L.J., Scarth, R., Mag, T., Ward, K., Adolph, D. Canola Oil i: Hui, Y.H. (Ed.) Bailey’s Industrial Oil & Fat Products, Edible Oil & Fat Products: Oils and Oil Seeds. volyme 2 (1996). USA: John Wiley & Sons, Inc.
- Fahey, J.W., Zalcmann, A.T., and Talalay, P. ”The chemical diversity and distribution of glucosinolates and isothiocyanates among plants”. *Phytochemistry* 56, 1 (2001): 5–51.
- ”FAO - News Article: 2050: A third more mouths to feed”. [Acceded: 21 december 2017]. http://www.fao.org/news/story/en/item/35571/icode/.
- Farhoosh, R., Einafshar, S., and Sharayei, P. ”The effect of commercial refining steps on the rancidity measures of soybean and canola oils”. *Food Chemistry* 115, 3 (2009): 933–38.
- Grosser, K. and van Dam, N. M. (2017). A straightforward method for gucosinolate extraction and analysis with high-pressure liquid chromatography (HPLC). *Journal of Visualized Experiments*.

- Gunstone, F. D. (2004) *Rapeseed and canola oil: Production, processing, properties and uses*. CRC Press.
- Hanschen, F.S., Herz, C., Schlotz, N., Kupke, F., Bartolomé Rodríguez, M.M., Schreiner, M., Rohn, S., and Lamy, E. "The Brassica epithionitrile 1-cyano-2,3-epithiopropane triggers cell death in human liver cancer cells in vitro". *Molecular Nutrition & Food Research* 59, 11 (2015): 2178–89.
- Hanson, S. R., Best, M.D., and Wong, C-H. "Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility". *Angewandte Chemie International*, 43 (2004): 5736–63.
- Herzallah, S., and Holley, R. "Determination of sinigrin, sinalbin, allyl- and benzyl isothiocyanates by RP-HPLC in mustard powder extracts". *LWT - Food Science and Technology* 47, 2 (2012): 293–99.
- Ivarson, E, Ahlman, A ,. Lager, I., and Zhu, L-H. "Significant increase of oleic acid level in the wild species lepidium campestre through direct gene silencing". *Plant cell reports* 35, 10 (2016): 2055–63.
- Lazzeri, L., Tacconi, R., and Palmieri, S. "In vitro activity of some glucosinolates and their reaction products toward a population of the nematode Heterodera schachtii". *Journal of Agricultural and food chemistry* 41, 5 (1993): 825–829.
- Lewandowski, I., Scurlock, J.M.O., Lindvall, E., and Christou, M. "The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe". *Biomass and Bioenergy* 25, 4 (2003): 335–61.
- Madawala, S. R.P., Kochhar, P., Dutta, P.C. Lipid components and oxidative status of selected specialty oils. *Grasas y Aceites,* 2 (2012).
- Mellon, F. A., Bennett, R. N., Holst, B. & Williamson, G. (2002). Intact glucosiolate analysis in plant extracts by programmed cone voltage electrospray LC/MS: Performance and comparison with LC/MS/MS methods. *Analytical Biochemistry*, 306(1), (2002): 83–91.
- Merker, A, Eriksson, D., and Bertholdsson, N-O. "Barley yield increases with undersown Lepidium campestre". *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* 60, 3 (2010): 269–73.
- Millán, S.M. Sampedro, C., Gallejones, P., Castellón, A., Ibargoitia,M.L., Aranzazu Goicolea, M., and Barrio, R.J. "Identification and quantification of glucosinolates in rapeseed using liquid chromatography–ion trap mass spectrometry". *Analytical and Bioanalytical Chemistry* 394, 6 (2009): 1661–69.
- Mistra Biotech Annual Report 2013 [Online] Available from: http://www.mistra.org/download/18.765ed0b6143735374811f0b/1473225430488/MistraBiotech_AR 2013_WEB.pdf [Accessed 2017-05-9]
- Nilsson, P., Johansson, S-Å,. and Merker, A. "Variation in seed oil composition of species from the genera Barbarea and Lepidium". *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* 48, 3 (1998): 159–64.
- Paunović, D, Šolević-Knudsen, T., Krivokapić, M., Zlatković,B,. and Antić, M. "Sinalbin degradation products in mild yellow mustard paste". *Hemijska industrija* 66, 1 (2012): 29–32.
- Polat, U. "The Effects on metabolism of glucosinolates and theirs hydrolysis products". *J. BIOL. ENVIRON. SCI., 4(10), (2010), 39-42.*
- Sandelius, S. "Den naturliga variationen av fettkomponenter I Lepidium campestre" Available from: https://stud.epsilon.slu.se/10094/1/sandelius_s_170411.pdf
- Shapiro, T.A.,. Fahey, J.W., Wade, K.L., Stephenson, K.K., and Talalay, P. "Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans". *Cancer Epidemiology and Prevention Biomarkers* 10, 5 (2001): 501–8.

- Sigma-Aldrich. Sulfatase from <I>Helix pomatia</I> S9751. Available: https://www.sigmaaldrich.com/catalog/product/sigma/s9751. [2017-11-20]
- Simpson, B.B., Ogorzaly, M.C. (2001) Economic botany. Third edition, McGraw-Hill Book Co, Singapore. ISBN 0-07-118188-1.
- Song, L. & Thornalley, P. J. Effect of storage, processing and cooking on glucosinolate content of Brassica vegetables. *Food and Chemical Toxicology*, 45(2), (2007): 216–224.
- Thies, W. ”Isolation of sinigrin and glucotropaeolin from cruciferous seeds”. *Lipid / Fett* 90, 8 (1988): 311–14.
- Tsao, R., Yu, Q., Potter, J., and Chiba, M. ”Direct and simultaneous analysis of sinigrin and allyl isothiocyanate in mustard samples by High-Performance Liquid Chromatography”. *Journal of Agricultural and Food Chemistry* 50, 17 (2002): 4749–53.
- Wretblad, S and Dixelius, C. ”B-genome derived resistance to Leptosphaeria maculans in near isogenic Brassica napus lines is independent of glucosinolate profile” *Physiologia Plantarum december 2000.*

5.2 Figures

- Figure 1. “Field cress” (Wikipedia), [acceded 14 November 2017] Available at: https://sv.wikipedia.org/wiki/F%C3%A4ltkrassing#/media/File:Lepidium_campestre2_W.jpg
- Figure 2. ”Glucosinolate”. *Wikipedia*, [acceded: 13 November 2017] Available at: https://en.wikipedia.org/w/index.php?title=Glucosinolate&oldid=810035163.

- Figure 3. ”Sinigrin hydrate”. *PubChem,* [acceded 21 November 2017] Available at: https://pubchem.ncbi.nlm.nih.gov/compound/23693101.

- Figure 4. ”Sinalbin”. *PubChem,* [acceded: 21 November 2017] Available at: https://pubchem.ncbi.nlm.nih.gov/compound/9601115.

- Figure 5. ”Reaction”. *Google,* [acceded 13 November 2017] Available at: http://homepage.ruhr-uni-bochum.de/Markus.Piotrowski/Bilder/GSBreakdown.gif

Acknowledgements

I would like to thank my supervisors Annica Anderson and Lena Dimberg.
Lena brought this project to my attention and it has been one of the most interesting project I have ever worked on. Also, big thanks for helping with the HPLC machine and theoretical support throughout the experiment.
Thank you to Annica that helped me in the lab with everything from finding my way to figuring out steps for a continuing method development. It has been a lot of fun working with you both.
Thanks to my family and friends for your support and motivation during this whole process. An extra big thank you to my sister Josephine for helping me throughout the experience.
Working in this project has been very rewarding and I have gained a lot of knowledge regarding the subject and lab work, I am very grateful for the opportunity to work with this project and hope that my discoveries might contribute with useful information for future developments.

Appendix 1: Popular summary

What types of glucosinolates exist in field cress seeds? Is there any variation between seeds from different countries?

Field cress is today not a domesticated crop for the publics consumption. Field cress belongs to the same family as rapeseed and both are producing oil, having high levels of linolenic acid and erucic acid which in a health and economical aspect is not attractive. From a farmer's point of view field cress has several benefits, it has a better cold tolerance than rapeseed which means that it can be grown further north, it's also a perennial crop which means that it can be grown together with an annual crop and therefor avoid tillage and minimise nitrogen leaching. Saving money for the farmer and minimises the strain on the environment. However, filed cress has some properties that makes it unattractive for food and feed. In field cress, there is a high amount of glucosinolates which when reacted with the enzyme myrosinase creates a pungent smell called mustard bomb. It's believed that glucosinolates that exist in field cress may have some anticarcinogenic properties. However, the amount of glucosinolates that is needed to achieve this is not known today. What is known is that glucosinolate exist naturally in the whole plant but in higher amount in the seeds. The mustard bomb is something that can be attractive in some food products for the taste however when it comes to feed its not deemed attractive.

In this study, a method for analysis of glucosinolates in field cress was developed, this was done using a trial and error method using the results from previous testing to move forward in the method development. The degradation of glucosinolates during storage of milled field cress seeds in a refrigerator for the duration of two weeks was also studied. Finally, 22 different field cress seed samples from three different countries were analysed to find out the amount of glucosinolates and what type of glucosinolates that exist in the seed.

It was found that in all of the samples tested, the glucosinolate sinalbin was the dominant one. This was done by comparing the result to another study. It was also found that the glucosinolates were degraded in the milled seeds when the samples were kept in the refrigerator, during the two weeks of storage the GSL content decreased with 55-36% which is quite big of a degradation. This has to be taken into consideration in future studies. An easy solution is to grind the sample just before analysis or to keep it in the freezer and only thaw it before use.

Today its known that glucosinolates exist in the seeds of field cress however the levels are too high to make it attractive. This could be solved by using traditional

breeding programs or by using gene modifications which has been done on rapeseed to make it to the product that is used today (canola oil). Similar to field cress, rapeseed had problems with its fatty acid profile and glucosinolate content but thanks to several breeding programs and gene modifications its today a healthy alternative with attractive attributes.

Throughout the method development several steps have both been taken away and been added on to make the method more reliable, cheaper and faster. The main steps being sulfatase treatment and sinigrin standard curve instead of internal standard that affects the result the most. The results also showed that even if the field cress seeds came from different countries there was no significant difference in the glucosinolate amount.

Even though the method is not yet reliable it has come a long way and can with some future changes hopefully be successful.