Glucosinolates and Glycoalkaloids as Biopesticides

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Authorization to Submit Thesis

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Abstract

Development of alternative pest control methods is a pressing issue as more species of pests develop resistance to synthetic pesticides and public demand for organically grown produce increases. Our research is focused on development of biopesticides from plant glycosides such as glucosinolates found in the family Brassicaceae and glycoalkaloids found in the genus *Solanum*. To achieve this goal, we use bioassay-guided methods for extraction and isolation of biologically active compounds from plant material. We then fractionate and analyze plant extracts with the highest pesticidal activity using chromatography coupled with mass spectrometry. The use of time of flight mass spectrometry allows for the accurate mass assignment and in silico compound identification. For example, we have identified several glycoside hydrolysis products in *Brassica juncea* and *Solanum sisymbriifolium* that exhibit nematicidal activity. We have also demonstrated that at least two different compounds are responsible for herbicidal activity of *Sinapis alba* towards three model weeds. Finally, we designed a bioprocessing pilot plant for efficient and cost-effective production of biopesticide formulations.

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Dedication

I dedicate this thesis to my grandmother, Ina Potaškina (1932-2019). Ina Sidorovna Potaškina (née Samsonova) was born and raised in Rosochaty Róg, Poland in a small Russian speaking Old-Believer Orthodox community of farmers that was established in the early 18th century. During the World War II, according to the Molotov–Ribbentrop Pact, her family was forced to relocate to the newly formed Lithuanian Republic of the U.S.S.R, where they were settled in the small village of Maišiagala. During the relocation, farms, animals, and personal belongings were confiscated alienating families from agriculture and forcing them to take any available often low wage jobs to support themselves. With many children, limited job opportunities, and depleted resources due to the war, families suffered. My grandmother's childhood was cut short as she had to start working alongside adults when she was just 11 years old. Her whole life my grandmother worked several jobs to first help her parents and siblings, and then to support her own family. In 2007, according to the international pledge on Property Restitution in Central and Eastern Europe, my grandmother was compensated by one hectare of agricultural land near Veiviržėnai, Lithuania, a small fraction of what was lost. At the age of 68 and wheelchair bound, she was not able to enjoy her land, but she had great hopes for me to become a farmer and take care of her land. While my grandmother never had an opportunity to obtain a proper education and could not relate to all the science I am doing, she understood well the value of soil and was proud of me pursuing degree in agriculture. My grandmother passed away in April 2019, at the age of 86. She lived a long life surrounded by family and friends, but the memories of her growing up on the family farm in Poland were her happiest memories.

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Chapter 1: Plant Based Pesticides in Agriculture

Need for Alternative Pest Management Strategies

Modern agriculture relies heavily on the use of pesticides to protect crops. It is extremely important to prevent yield losses as the global demand for food is growing steadily. It is estimated that by 2050 the Earth's population will be close to 10 billion (Heap, 2014). At the same time, the area of land with productive soil is shrinking rapidly due to the losses in soil fertility, competition for land use with other industries, and urbanization. Thus, higher crop yields need to be produced on the smaller acreage. To make the situation worse, it is estimated that more than 600 species of pests have developed resistance to commonly used pesticides such as imidazolinones, sulfonylureas, and triazolopyrimidines, and sulfonanilides that are routinely used in agriculture to protect crops (Ferguson et al., 2001; Heap, 2014; Hoy, 1998). As a result, currently available pesticides are becoming obsolete as pests develop resistance mechanisms against them (Bernards et al., 2012). Consequently, producers resort to more toxic but still efficient pesticides. However, many of these last resort pesticides are being phased out due to detrimental effects on the environment and human health. For example, 1,3-dichloropropene can cause cancer in humans and as a result is currently in the process of being phased out in the European Union despite it being one of few pesticides that is still effective against potato cyst nematodes, *Globodera rostochiensis* and *Globodera pallida*, major potato pests that causes an estimated annual crop loss of $78 billion worldwide (Lima et al., 2018). Another group of last resort pesticides, chlorofluorocarbons such as methylene bromide, have been linked to depletion of the ozone layer and, as a result, are banned in many countries including the U.S.A. The combination of the rise in pesticide resistance and decrease in the number of available pesticides creates a

void in available pest management options in agriculture. There is thus a strong need for development of alternative pest management strategies (Shaner and Beakie, 2014).

Botanical Pesticides in Organic Agriculture

One approach in the development of alternative, economically sound methods of pest control is the use of plant-based pesticides. The history of using plant-based compounds as pesticides dates to 100 BC when aromatic plants were used as insect repellants (Isman, 2006). Since that discovery, dried plants, plant oils, tinctures, and plant extracts from a variety of plants have been used across the world to protect crops from pests (Pavela, 2016). For example, extracts from tobacco leaves have been used to control plum beetles and extracts from *Derris elliptica* roots have been used against weeds and insects (Gnany, 2012). In Africa and the American tropics, *Jatropha curcas* has been widely used for pest control (Ntonifor et al., 2010). In many parts of Africa, indigenous plants like *Piper guineense* and *Tephrosia vogelii* have been traditionally used for insect control (Ogendo et al, 2003; Bekele et al, 1997). However, after World War II, with the advance of science and technology and the development of cheap synthetic pesticides, the use of botanical pesticides became limited (Ware and Whitacre, 2004). It is only recently, due to the increase in public awareness of environmental and health related issues associated with the use of synthetic pesticides, that botanical pesticides are making a comeback. Specifically, botanical pesticides have received wide use in organic farming as they can be classified as biopesticides for regulatory purposes (EPA, 2012). Organic agriculture is a relatively small segment of the agricultural market. In the United States, organic produce accounts for about 4% of total sales (USDA, 2017) and in the European Union organic agriculture accounts for 7% of total agricultural lands (Erostat,

2019). However, organic agriculture is rapidly growing as the public demand for organic food is increasing and becoming mainstream (Lester, 2006). More consumers are choosing organically over conventionally grown produce due to perceptions that organic food is safer, healthy, and environmentally friendly (Lester, 2006; Connor, 2008). Still, organically grown produce is economically disadvantaged due to the cost associated with certification and production (Connor, 2008). The cost and availability of pest management in general and biopesticides in particular constrain improvements in organic agriculture (Ross and Lembi, 1999). Plant-based biopesticide production is limited by manufacturing technologies, storage, and viable application methods as well as availability of plant feedstocks. In many cases, there is simply not enough plant material to make biopesticides commercially viable.

Biopesticide Production

The pesticidal activity of plants is due to the action of secondary metabolites that are part of the plant's defense system (Miresmailli and Isman, 2014). It is believed that biological activity of these secondary metabolites is a result of plants' co-evolution with pests (Duke et al., 2002). As the result, these plants have developed the capacity to produce compounds that have biological activity. One or several compounds with biological activity toward pests can be present in a plant. For example, neem, *Azadirachta indica*, contains more than 100 biologically active compounds including terpenoids, carbohydrates, proteins, and sulfa compounds, many of which exhibit pesticidal properties (Schmutterer, 1990). Azadirachtins, margosonic acid, margosopicin, nimbinin, nimbosterol, nimbocinone, nimbidin, nimbinin, and aldobiuronic isolated from different parts of neem have been used as pesticides, and becoming prototypes for synthetic analogues and building blocks for synthetic

pesticides. Currently, more than 1079 plant species worldwide were identified to contain at least one pesticidal compound (Prakash and Rao, 1997). These plants are often used in modern and indigenous agriculture. For example, *Brassicaceae* plants have been widely adopted as a cover crop and rotation crop in modern agriculture for control of weeds and insects in the United States and Australia (Brown and Morra, 1997; Kirkegaard and Matthiessen, 1998). Other plants, such as *Blumea eriantha* are only used locally by small acreage farmers in Eastern and Western Ghats and South India (Dongre and Rahalkar, 1982). The limited usage of these plants can be due to the specific conditions needed for plant growth such as temperature and moisture regime, slow plant growth, or limited amount of plants for wider adaptation. While cultivation and use of plants with pesticidal properties as cover and rotation crops may not be possible, the use of biopesticides prepared from these plants is still an option.

In the simplest form, biopesticides can be applied as dried or pelletized plant materials (Zasada et al., 2009). For example, meal of mustard has been used in the form of flakes or pellets in the management of pests in organic strawberries (Seigies and Pritts, 2006). This approach requires minimum processing and preparation of the biopesticide and can be implemented as a part of crop management. However, batch-to-batch variation in the concentration of pesticidal compounds in the plants can be significant due to the plant variety, growth season variation, and other factors, thus creating challenges for achieving consistent application rates for efficacy. For example, concentration of the pesticidal compound karanjin, in the cake of *Pongamia pinnata* can vary from 0.01 to 0.19% (Prabhu et al., 2002). Similarly, the concentration of glucosinolates in mustard meal pellets can vary by 27% from season to season (Farm Fuel Inc, personal communication). To avoid this

inconsistency when using plant-based pesticides in pest management, concentration of the active ingredient must be carefully monitored prior to biopesticide application. However, it may be challenging for an end user to do so as analysis of plant-based pesticides are not routinely performed in commercial analytical labs.

Another way of utilizing pesticide-producing plants for pest management is to prepare a biopesticide by extracting, concentrating, or isolating the active compound, thereby maintaining consistency of the final product. The added benefit is to reduce the amount of bulk material and undesired components that have no pesticidal influence or negatively influence efficacy. Although isolation of pesticidal compound is the best option to ensure formulation consistency, possible negative consequences include decreased efficacy, increased cost, and increased regulatory barriers for EPA registration. However, biopesticidal activity may be synergistic with more than one compound that may be the reason for the increase in observed effect. Thus, when only one of biologically active compound is targeted for isolation, a decline in activity relative to the whole extract will be observed. In addition, viability of extracting the active ingredient may be cost prohibitive and the process itself maybe result in a product unacceptable for organic certification. For example, if isolation requires the use of harsh organic solvents such as dichloromethane or hexane, extraction costs will increase and organic certification of a final product will be problematic.

Crude and purified plant extracts are other options for production of biopesticides. Unlike isolation, extraction is aimed at concentrating the pesticidal compounds rather than separating them. As a result, while the concentration of the active compounds is increased and the majority of the plant matrix is removed, extracts still contain other plant constituents. In some cases, co-extracted compounds can be considered as beneficial. For example, when

extract of *Sinapis alba* is prepared for use as a herbicide, other components such as polyphenolic compounds and salicylic acid derivatives are co-extracted (Dubie et al., 2012). These compounds have no pesticidal effect, but they exhibit antioxidant activity and can act as stabilizers of the prepared extract. Crude extracts can further be purified and/or concentrated to eliminate undesirable constituents and increase concentrations of the active ingredient. Matrix components that have no negative effect on the overall performance of the biopesticide are left in the extract. This could result in lower cost of the final product production as isolation of the active compound is not required. Still, the overall cost including shipping charges, possible higher application rates, and stability of the extract needs to be considered to assure the economical viability of the product.

However, in some cases, co-extraction of other chemicals from the plant material is undesired. For example, seeds of oil crop *Jatropha curcas* contain high concentrations of glucosinolates that can potentially be used for biopesticide production (Makkar et al, 1997). However, the seeds are also reach in a toxalbumin called curcin, ricin, and cyanic acid that are toxic to humans and animals (Singh et al., 2010). Thus, using a crude extract of the whole plant is not acceptable as a biopesticide.

Chapter 2: Glucosinolates as Natural Pesticides

Introduction

Properties and Occurrence of Glucosinolates

Glucosinolates are defined as thioglucosides that contain a sulfated aldoxime with an amino acid derivative side group (Fig. 1) (Agerbirk and Olsen, 2012; Clarke, 2010). More than 200 side groups were identified making glucosinolates a diverse group of compounds. Glucosinolates are present in a wide range of plant, predominantly in the Brassicaceae such as broccoli, cauliflower, radishes, and mustards (Hwang et al., 2019). The concentration and type of glucosinolates vary significantly among the plant species as well as within the plant, plant growth stage, and plant stress level (Kushad et al., 1999). For example, for two mustard species *Brassica juncea* and *Sinapis alba,* the distribution and type of glucosinolates were different in shoots, roots, and seeds (Fig. 2) (Popova, 2019 unpublished results). Specifically, the concentration of sinalbin, the major glucosinolate in *S. alba,* is five times higher in seed than in shoots and nine times higher in seed than in roots. The concentration of sinigrin in *B. juncea* seeds is two times higher than in shoots and fifteen times higher than in roots. The distribution of glucosinolates in the two mustards also varies with the plant part, with the seed predominately containing one major glucosinolate and shoots and roots having equal distribution of several glucosinolates. Similar trends were observed for different plants as well, demonstrating the dynamic nature of glucosinolate composition as affected by a variety of factors (Porter et al., 1991).

Glucosinolates themselves display very little biological activity. However, in the presence of the endogenous enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147)

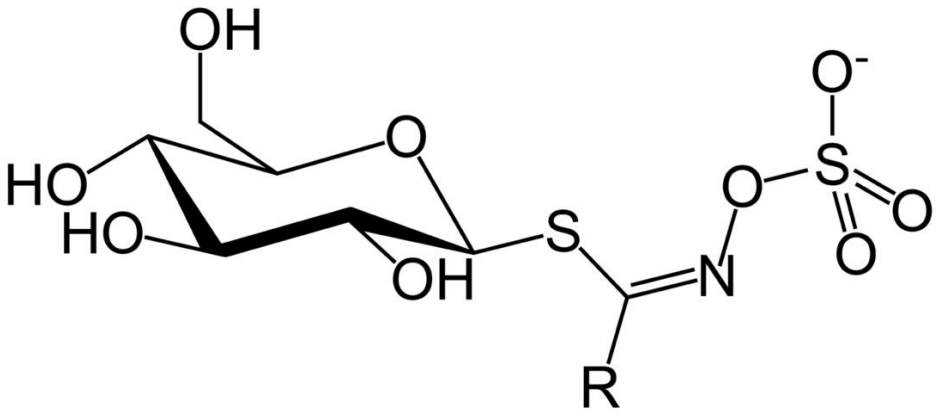

Figure 2.1. General structure of glucosinolates, where R is a substitute group.

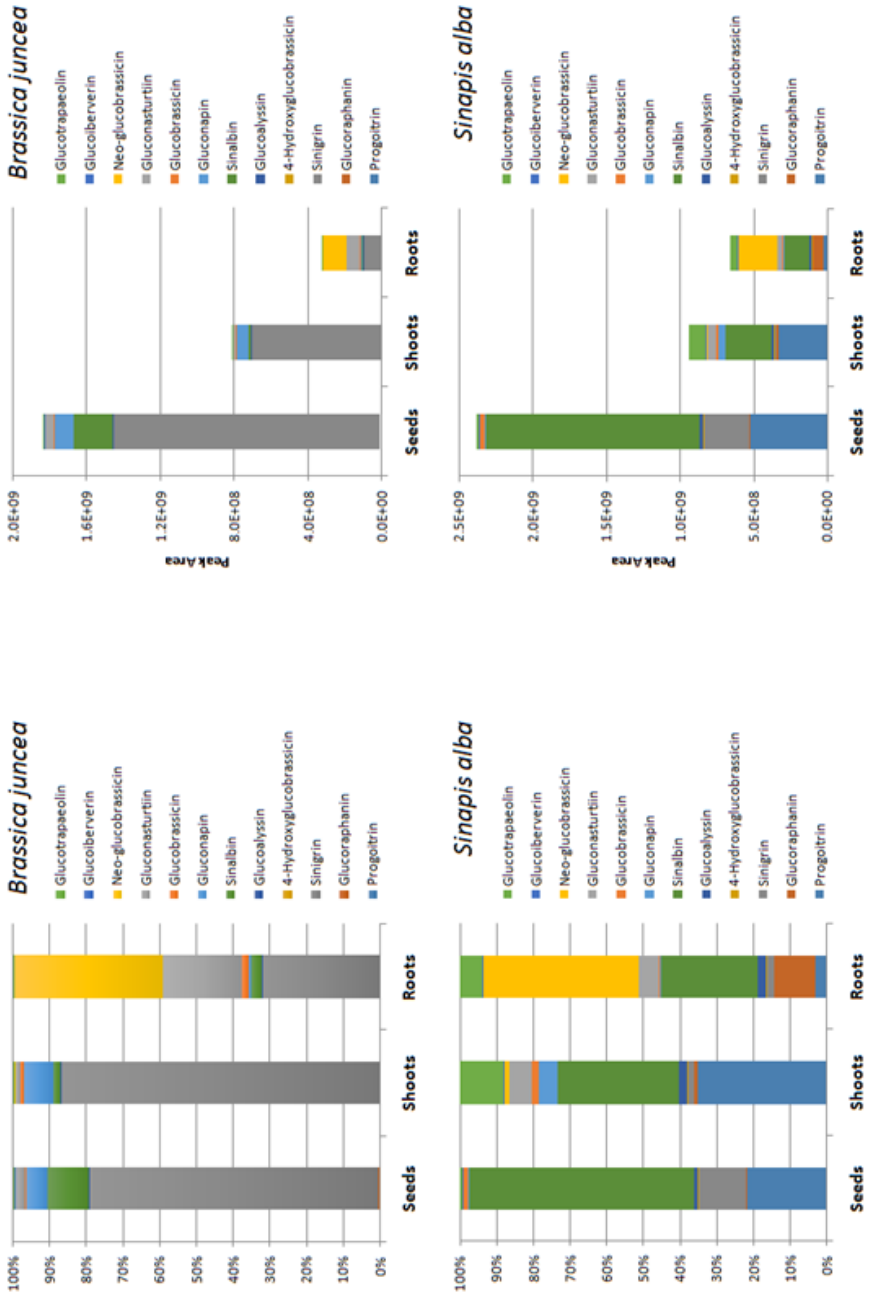

Figure 2.2. Distribution of glucosinolates in *Brassica juncea* and *Sinapis alba* mustards (Popova et al., unpublished data).

and moisture, glucosinolates are enzymatically hydrolyzed and biologically active compounds are formed (Brown and Morra, 1997; Rosa et al., 1997). Depending on the original structure and hydrolysis conditions, products include isothiocyanates, ionic thiocyanate, nitriles, or oxazolidinethiones (Fig. 3) (Borek et al., 1994; Brown and Morra, 1995; Morra and Kirkegaard, 2002). While myrosinase is also present in glucosinolate-containing plants, the enzyme is stored in plant cell vacuoles and is physically segregated from glucosinolates (Bending and Lincoln, 1999). This intercellular separation of glucosinolates and enzyme explains the absence of glucosinolate hydrolysis in intact plants (Brown and Morra, 1997). However, when plant tissue is physically disrupted in the presence of moisture by, for example, insects or animal feeding, glucosinolate hydrolysis is activated and glucosinolate hydrolysis products are produced (Bending and Lincoln, 1999; Borek and Morra, 2005).

Unlike glucosinolates, glucosinolate hydrolysis products have a range of biological properties (Brown and Morra, 1997; Rosa et al., 1997). They can impart flavors to foods (D'Antuono et al., 2009), function as anticarcinogens in human nutrition (Hayes et al., 2008), or can be used as broad-spectrum antimicrobials to extend the shelf life of various food products (Dai and Lim, 2014). In agriculture, glucosinolate hydrolysis products exhibit insecticidal, nematicidal, and herbicidal properties (Brown and Morra, 1997; Earlywine et al., 2010; Stevens et al., 2009, Morra et al., 2018).

Seeds of mustards such as *B. juncea* and *S. alba* have been documented to have the highest concentrations of glucosinolates among *Brassica* species ranging from 90 to 202 μmole/g^{-1} dry weight (Popova and Morra, 2014; Morra et al., 2018). Mustard seeds also contain up to 40% of oil by weight (Snyder et al., 2009) which, when extracted is used in

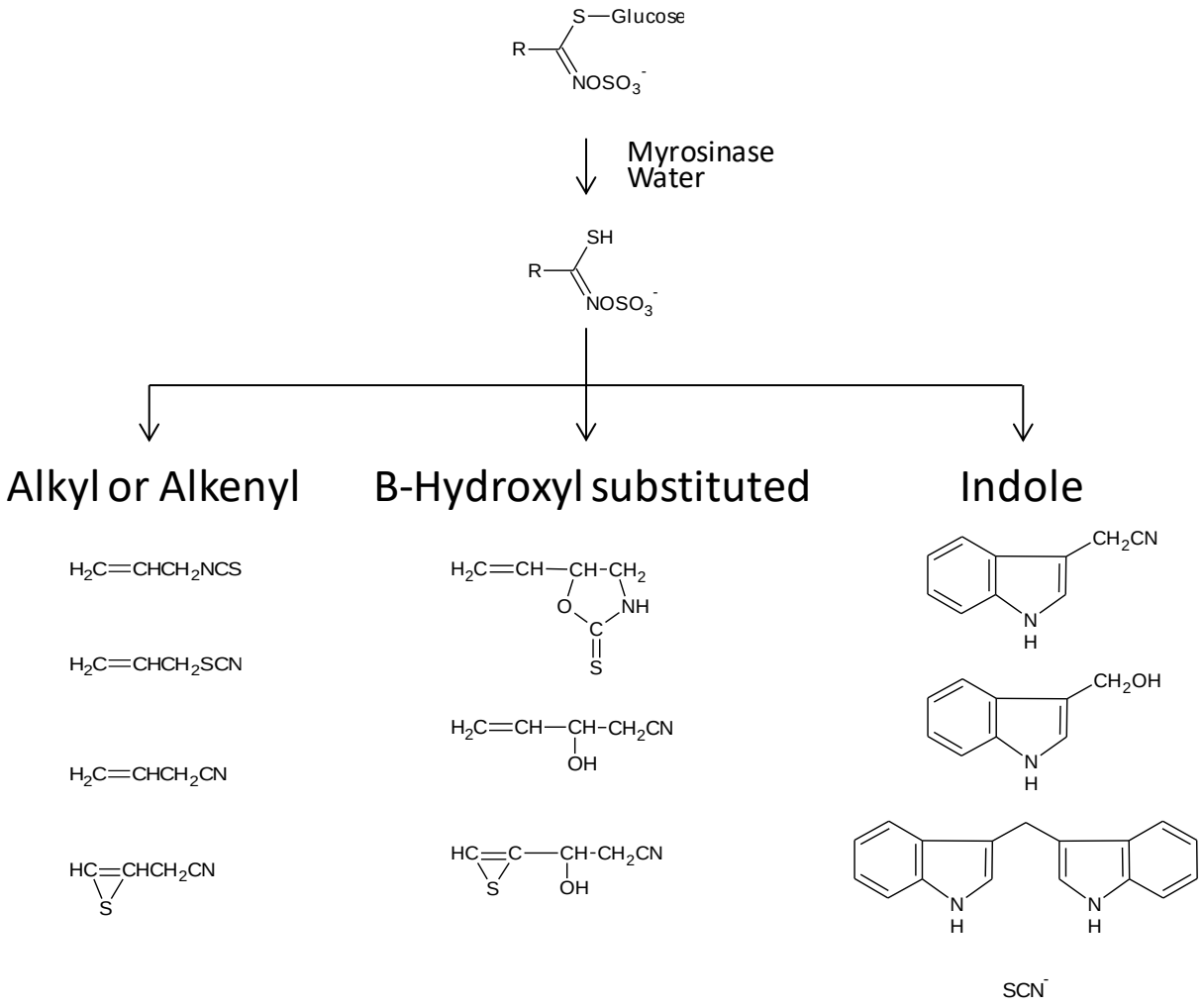

Figure 2.3. Enzymatic hydrolysis of glucosinolates. Depending on the original structure and hydrolysis conditions, isothiocyanates, ionic thiocyanate, nitriles, or oxazolidinethiones.

products such as emulsifiers, industrial-grade oil, and biodiesel (Clayton, 2009). The mustard meal left after oil extraction can contain up to 6% nitrogen, has a C:N ratio of ~ 8:1, and contains up to 40% of crude protein content making it suitable for animal feed (Burow et al., 2007). However, the presence of glucosinolates prevents it from being used because glucosinolate degradation products are goitrogenic to animals (Burow et al., 2007). Extraction of glucosinolates from mustard meals can resolve that problem by producing a glucosinolate rich extract that can be used in pest control as well as producing a detoxified meal which can be used as a high nutrient animal feed (Morra et al., 2017).

Extraction of glucosinolates

Glucosinolates can be extracted from the plant matrix using different extraction techniques with different solvents (Table 1) (Popova and Morra, 2014). For extraction of intact glucosinolates in aqueous solvents or with water, special care should be taken to avoid conditions that favor enzymatic hydrolysis. Myrosinases, represented in plant material by a various isoemzymes, have pH optima in the range of 5 to 7 (Durham et al., 1990; Bjorkman et al., 1973). Temperatures higher than 60 °C have been shown to deactivate myrosinase (Iori et al., 1996). Thus, the methods for intact glucosinolate extraction should have pH and/or temperatures adjusted outside these regions. The use of organic solvents has been shown to have inhibitory effect on myrosinase and its use is favorable for intact glucosinolate extraction. The most common solvents for glucosinolate extraction are methanol and ethanol as they provide good extractability and are relatively inexpensive and safe to use.

Table 2.1. Extraction of glucosinolates from mustard seeds.

Species	Solvent	Extraction conditions	Reference
Brassica juncea , Sinapis alba	73% Aqueous methanol	1 h at room temperature shaking at 1:55 (w:v, meal to solvent ratio)	Popova and Morra, 2014
	73% Aqueous methanol	1 h at room temperature shaking at 1:55 (w:v, meal to solvent ratio)	Popova and Morra, 2014
Brassica juncea , Sinapis alba	70% Aqueous methanol	10 min at 75 °C shaking at 1:49.5 (w:v, meal to solvent ratio)	Doheny-Adams et al., 2017
	70% Aqueous methanol	10 min 75 °C shaking at 1:49. 5 (w:v, meal to solvent ratio)	Doheny-Adams et al., 2017
Brassica rapa	80% Aqueous methanol	30 min at room temperature shaking at 1:50 (w:v, meal to solvent ratio)	Ishida et al., 2011
Brassica juncea, Sinapis alba	Water	10 min at 100 °C, then 4 h at 70 °C shaking at 1:250 (w:v, meal to solvent ratio)	Herzallah and Holley, 2012

Cold shaking, water reflux, and ultrasound are used for extraction of glucosinolates from seed meal (Tsao et al., 2002; Cools and Terry, 2012; Kaushik and Agnihotri, 1999; Gerendas et al., 2009; Wang et al., 2010; Dubie et al., 2013). For example, when 50% aqueous acetonitrile was used as a solvent to extract sinigrin from mustard meal, boiling was necessary to achieve 99.8% recoveries (Tsao et al., 2002), whereas extraction at room temperature resulted in ~20% degradation of sinigrin. It was demonstrated that the use of heated 70% methanol rather than 50% acetonitrile resulted in the greatest sinigrin recovery (Cools and Terry, 2012; Gerendas et al., 2009) Investigators have found that extraction with 80% cold methanol was optimal (Doheny-Adams et al., 2017), whereas boiling mustard meal with 100% alcohol resulted in non-enzymatic hydrolysis of sinigrin (Cools and Terry, 2012). The use of ultrasonic assistance for extraction of sinigrin was demonstrated to improve sinigrin recovery (Wang et al., 2010). While results using all three methods were claimed to be satisfactory, direct comparison of the methods cannot be made as no absolute extraction efficiency was evaluated. The problem with evaluating any relative extraction method is the difficulty in obtaining a blank sample matrix for extraction of glucosinolates. Thus, reported extraction efficiencies are relative to the maximum achieved recoveries rather than absolute concentrations in seed meal. Comparison across the different laboratories is also problematic due to natural variation of glucosinolate concentrations in seeds and glucosinolate concentration changes during storage. For example, when mustard meal was stored at 4 °C for 6 months, glucosinolate degradation was observed (Morra and Borek, 2010).

The major advantages of using boiling water as an extractant is its low toxicity and low cost. However, achieving consistent temperatures and using an accurate meal to water ratio to maintain thermal capacity of water is crucial. The use of organic solvent-based

extraction procedures is more flexible because myrosinase inhibition prevents hydrolysis of glucosinolates. However, organic solvents present challenges associated with higher cost and the need for special handling and disposal of used organic solvents. Additionally, co-elution of other mustard seed chemicals needs to be considered when the extractant is selected. For example, *S. alba* mustard meal has a relatively large content of mucilage (up to 5% by weight of seed), which is a mixture of a pectic material consisting primarily of galacturonic acid, galactose, rhamnose, and a 1,4-linked β-d-glucan (Balke and Diosady, 2000; Cui et al., 1993). In aqueous media, mucilage expands when hydrated to form hydrocolloids (Gerhards and Walker, 1997). As a result, this can present challenges in filtering, separation of extracted meal, and incomplete extraction. Organic extractants reduce these problems, but co-extract more non-polar compounds that potentially affect glucosinolate quantification (Dubie et al., 2013).

Analysis of glucosinolates

Glucosinolates can be quantified by a range of analytical techniques (Clarke, 2010). However, one of the most often-used methods for glucosinolate analysis is quantification of desulfated glucosinolates by HPLC/UV (ISO, 1992). While this method is standardized and can be used across laboratories, it includes many sample treatment steps making it rather time consuming, meticulous, and prone to artifacts (Brown et al., 2003; Hennig et al., 2012; Ramallo et al., 2004). Recent advances in the use of hyphenated techniques such as high performance liquid chromatography coupled with mass spectrometry have resulted in the development of numerous methods for analysis of intact glucosinolates (Popova and Morra, 2013; Glauser et al., 2012; Gratacos-Cubarsi et al., 2010; Maldini et al., 2012; Mellon et al.,

2002; Tian et al., 2005). HPLC/MS has several obvious advantages such as shorter analysis times, higher sensitivity, and structural conformation from the mass spectrum. Unfortunately, the lack of analytical standards and analysis and instrumental maintenance costs are major shortcomings of HPLC/MS based methods.

Recently, two very successful methods for chromatography-based analysis of mustard glucosinolates were developed (Popova and Morra, 2013; Popova and Morra, 2012). The first method is based on the use of time-of-flight (TOF) MS (Popova and Morra, 2012). The inherent high mass determination accuracy of TOF MS allows for identification of glucosinolates in the absence of pure standards, while co-eluting matrix components can be monitored simultaneously under full-scan conditions.

The second successful method for analysis of glucosinolates is the use of anion chromatography (Popova and Morra, 2013). While ion chromatography (IC) is a less popular technique, its simplicity and low cost provide major advantages over more sophisticated techniques. Another significant advantage of IC is that hydrolysis products along with the intact glucosinolates are measured, thus quantifying directly active ingredient concentration rather than assuming complete hydrolysis and theoretical reaction stoichiometry. Accurate measurement of bioactive compounds is crucial for mustard meal use as a biopesticide. Overestimation of biopesticidal activity can occur when glucosinolate hydrolysis is incomplete due to low myrosinase activity or when glucosinolates are converted to other compounds during seed processing (Campbell and Slominski, 1990; Bell and Keith, 1991). As a result, this would lead to low and inconsistent efficacy of such glucosinolate containing products. Conversely, underestimation of biological activity occurs when glucosinolates are already partially decomposed in the mustard meal sample. For example, hydrolysis can occur

during improper storage of mustard meal such as at high temperature or at high relative humidity (Dai and Lim, 2014). In addition, simultaneous analysis of both glucosinolates and their respective hydrolysis products in mustard meal prior to its application provides a more accurate representation of potential biological activity, yielding information on the efficiency of meal-processing methods.

Methods and Materials

Plant material

Samples of mustard meal were prepared from locally obtained mustard seeds obtained from Northwest Pacific Northwest Farmers' Cooperative (Genesee, ID, USA). Several varieties of mustard were used throughout the study: Pacific Gold (*B. juncea*), Kodiak (*B. juncea*), and IdaGold (*S. alba*). These varieties were used due to the relatively high glucosinolate content as compared to other locally grown varieties (Brown et al., 1998; Davis et al., 2004). Oil was separated mechanically using a cold press (University of Idaho, Moscow, ID) as previously documented (Brown et al., 1991). Mustard meal produced as flakes was packed in seed bagged and stored in the dark, cool place. Prior to use, mustard meals were homogenized and passed through a 2-mm sieve.

HPLC/DAD/TOF-MS analysis of intact glucosinolates, 4-hydroxybezyl alcohol, and 2-hydroxybenzyl acetonitrile

HPLC analysis was performed using an Agilent 1200 Series HPLC with a diode array detector (DAD) coupled to an Agilent G1969A TOF-MS equipped with an ESI source (Agilent, Santa Clara, CA, USA). The chromatographic separation of intact glucosinolates

was performed using a Zorbax SB-Aq, 50 mm × 4.6 mm, 3.5 μm rapid resolution column equipped with a Zorbax SB-Aq, 12.5 mm × 4.6 mm, 5 μm guard column (Agilent, Santa Clara, CA, USA) maintained at 30 °C. The injection volume was 5 μL. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient program started with isocratic elution using 5% B for 3 min, followed by a linear gradient to 70% B from 3 to 10 min. For the first 2 min of the analysis, the flow was diverted from the MS to prevent MS contamination and ion suppression with salts and other polar species. The flow rate was 0.4 mL min^{-1} and spectra were recorded from 190 to 400 nm.

Electrospray ionization was operated in the negative mode. The absolute values for electrospray ionization potential and collision-induced dissociation potential were 3500 and 200 V, respectively. Gas temperature was 350 °C, drying gas (N_2) flow rate was 12 L min^{-1}, and nebulizer pressure was 2.4 × 105 Pa. The analyses were conducted in a profile mode within an m/z range from 100 to 1000 amu. Quantification was performed in the reconstructed ion current mode using m/z values of 358.03 (sinigrin) and 424.04 (sinalbin). 4-Hydroxybezyl alcohol and 2-hydroxybenzyl acetonitrile were quantified by monitoring absorbances from 220 to 230 nm. External calibration curves were used for quantification of all the analytes. Limits of detection were 0.11 for 4-hydroxybezyl alcohol and 0.21 mM for 2-hydroxybenzyl acetonitrile.

HPLC/UV analysis of 2-propenyl isothiocyanate

Derivatization of 2-propenyl isothiocyanate was performed prior to analysis. An aliquot (10–100 μL) of the hydrolysis mixture was obtained through a septum of a capped

glass reaction vial and diluted with methanol to 5 mL. Diluted solution (860 μL) was added to a 2-mL autosampler vial containing 860 μL of 100 mM potassium phosphate at pH 8.5. An additional 280 μL of 35 mM 1,2-benzenedithiol/1% mercaptoethanol in methanol was added, and the vial was capped and incubated for 1 h at 65 °C. After incubation, the mixture was vortexed, centrifuged at 800 g, and analyzed by HPLC/UV. Validation of analyte recovery was confirmed using aqueous samples spiked with 2-propenyl isothiocyanate.

Analysis of derivatized 2-propenyl isothiocyanate was performed using an Agilent 1200 Series HPLC system with a diode array detection (DAD) system on an Agilent XDB C18 (1.8 μm, 4.6 × 50 mm) column (Agilent, Santa Clara, CA, USA). The column was thermostated at 30 °C. Isocratic elution was used with 90% acetonitrile in water and the flow rate was 0.6 mL min^{-1}. Spectra were recorded from 190 to 400 nm in 2-nm steps. The injection volume was 5 μL and the runtime was 5 min with derivatized 2-propenyl isothiocyanate elution at 1.4 min. Derivatized 2-propenyl isothiocyanate was quantified at an extracted wavelength channel of 350–360 nm using an external calibration curve.

Ion chromatographic analysis of intact glucosinolates, sulfate, and ionic isothiocyanate

Sinigrin, sinalbin, sulfate, and SCN^- in the extracts were quantified by ion chromatography (IC) using a Dionex Ion Analyzer equipped with a GP40 gradient pump, ED40 electrochemical detector, and an AS40 autosampler as described previously (Popova and Morra, 2014). Briefly, a Dionex 4 × 210 mm Ion-Pac AS16 anion exchange column was

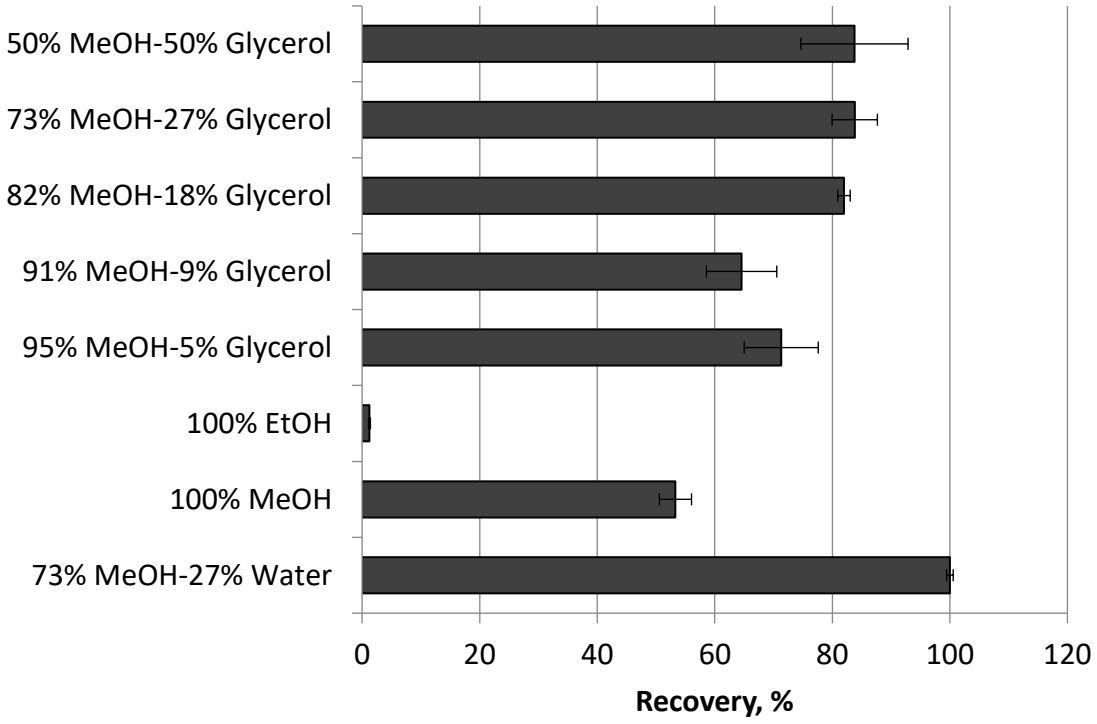

Figure 2.4. Extraction recovery of sinalbin from mustard seed meal from *S. alba*.

used for separation. Sodium hydroxide (100 mM) was used as the mobile phase at a flow rate of 0.9 mL min^{-1}. The detector stabilizer temperature was set at 30 °C with temperature compensation of 1.7% per °C. Anion suppressor current was set to 300 mA. The injection volume was 20 μL.

Results and Discussions

Extraction and hydrolysis of sinalbin from Sinapis alba

Sinalbin, a major glucosinolate of *S. alba*, can be extracted with a series of solvents (Fig. 4). Sinalbin is moderately soluble in aqueous solutions due the presence of a hydrophilic sulfate group. Additionally, it is soluble in organic solvents due to the hydrophobic interactions between the benzolic ring and organic solvent molecules. When a series of solvents was tested for quantitative recovery of sinalbin from *S. alba* mustard seed, the most acceptable results were achieved with methanolic based solvent systems where the percentage of methanol was above 50%. When the percentage of methanol was lower, recoveries were reduced as well. However, when pure methanol was used for extraction, poor recoveries were obtained. Similarly, pure ethanol was not an efficient solvent for sinalbin extraction. When a series of methanol-water solutions were used, a clear trend of sinalbin extractability was observed (Fig. 5). As anticipated when methanol concentration was less than 30% by volume, hydrolysis of sinalbin was observed. Consequently, no or very little sinalbin was recovered. However, increasing methanol concentration from 40 to 70% resulted in incomplete sinalbin hydrolysis likely resulting from myrosinase inhibition (Iori et al., 1996). As a result, extractability of sinalbin was increased. Further increases in methanol

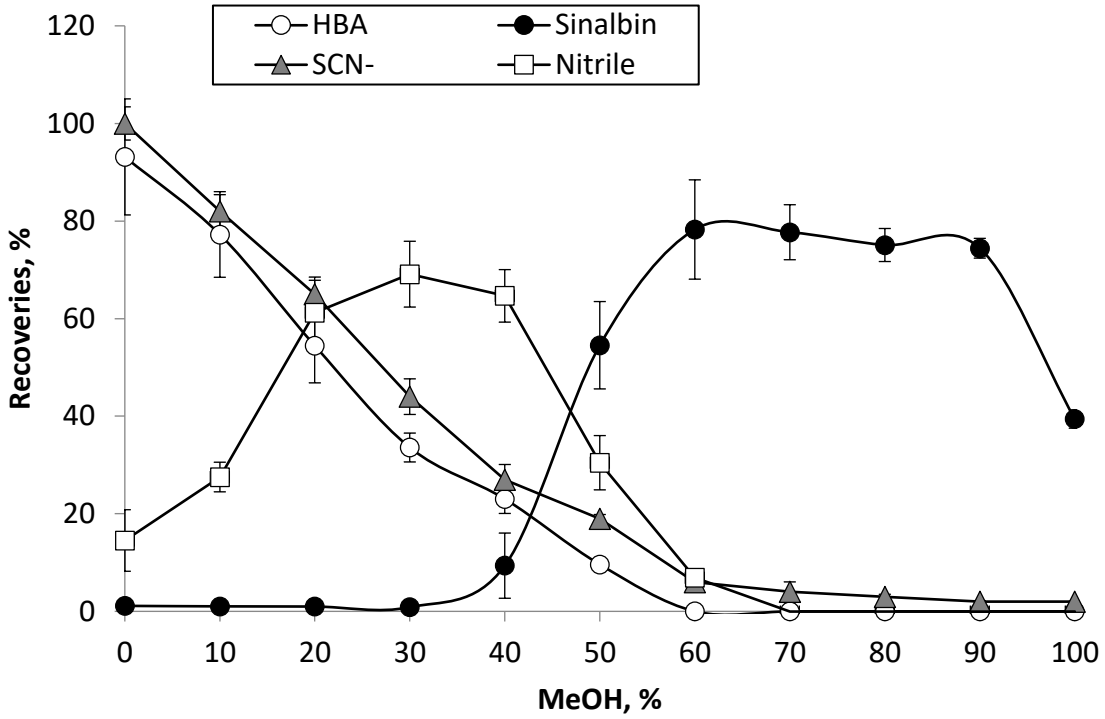

Figure 2.5. Recoveries of intact sinalbin and its hydrolysis products in different methanol-water solutions. Vertical bars represent standard deviations of the mean of three replicates.

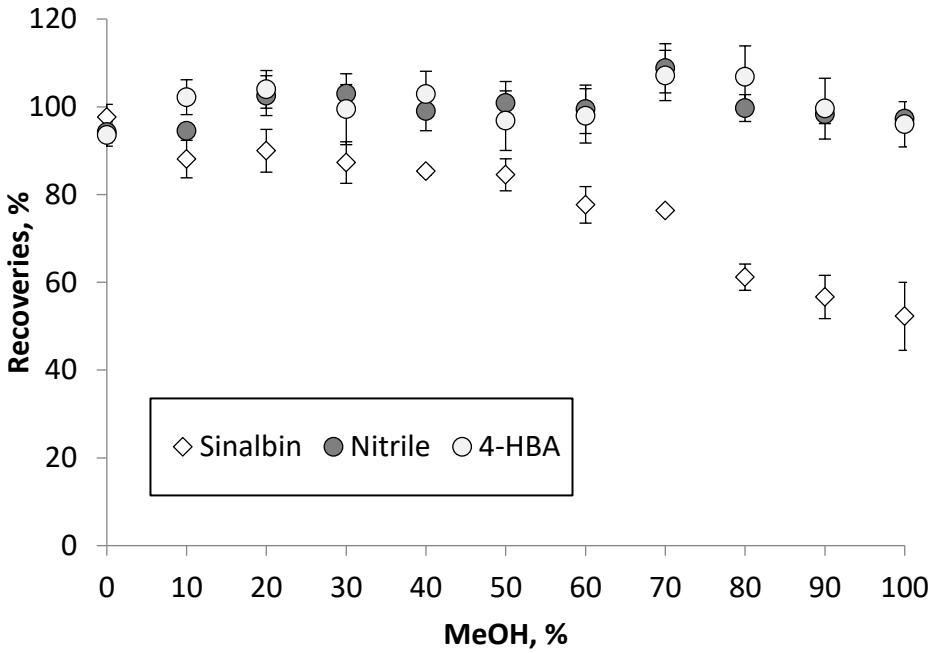

Figure 2.6. Extraction recoveries for intact sinalbin and its hydrolysis products from mustard seed meal using a series of methanol-water solutions. Vertical bars represent standard deviations of the mean of three replicates.

concentration led to complete deactivation of myrosinase and consequently, virtually no sinalbin hydrolysis was observed. However, extractability of sinalbin decreased significantly (Fig. 6). Hydrolysis products of sinalbin, 4-hydroxybenzyl alcohol and ionic thiocyanate, exhibited somewhat similar extraction trends from the mustard seed meal and were quantitatively recovered in pure aqueous solution. However, clear discrepancies between recoveries of 4-hydroxybenzyl alcohol, 4-hydroxybenzyl isothiocyanate, ionic thiocyanate and sinalbin were observed in 0 to 30% methanol extracts (Fig. 5). As shown previously, hydrolysis of sinalbin occurs with the formation of unstable thiohydroximate-*O*-sulfonate, which is then converted to 4-hydroxybenzyl isothiocyanate through Lossen rearrangement (Hanschen et al., 2014). 4-Hydroxybenzyl isothiocyanate is further converted to an unstable quinone methide intermediate that is primarily transformed to HBA (Borek and Morra, 2005). However, it can also form different products with protonated nucleophiles depending on reaction conditions (Toteva et al., 2011). Methanol addition can also interfere with rearrangement, stabilizing 4-hydroxybenzyl isothiocyanate in solution such that it cannot be further transformed into HBA. Thus, depending on the desired chemical isolation of the seed meal, different solvent composition should be used. If, for example, sinalbin is a target chemical for extraction, 70-80% aqueous methanol should be used for extraction. If ionic thiocyanate or 4-hydroxybenzyl alcohol are target extractants, solvents with less than 10% aqueous methanol should be used. Finally, if the objective of the extraction is 4-hydroxybenzyl isothiocyanate, the maximum extraction efficiencies could be achieved at ~30% aqueous methanol.

The use of different 30% aqueous alcohols as compared to water resulted in not only different absolute recoveries of 4-hydroxybenzyl alcohol, 4-hydroxybenzyl isothiocyanate, ionic thiocyanate and sinalbin, but also in differences in their relative distribution (Fig. 7).

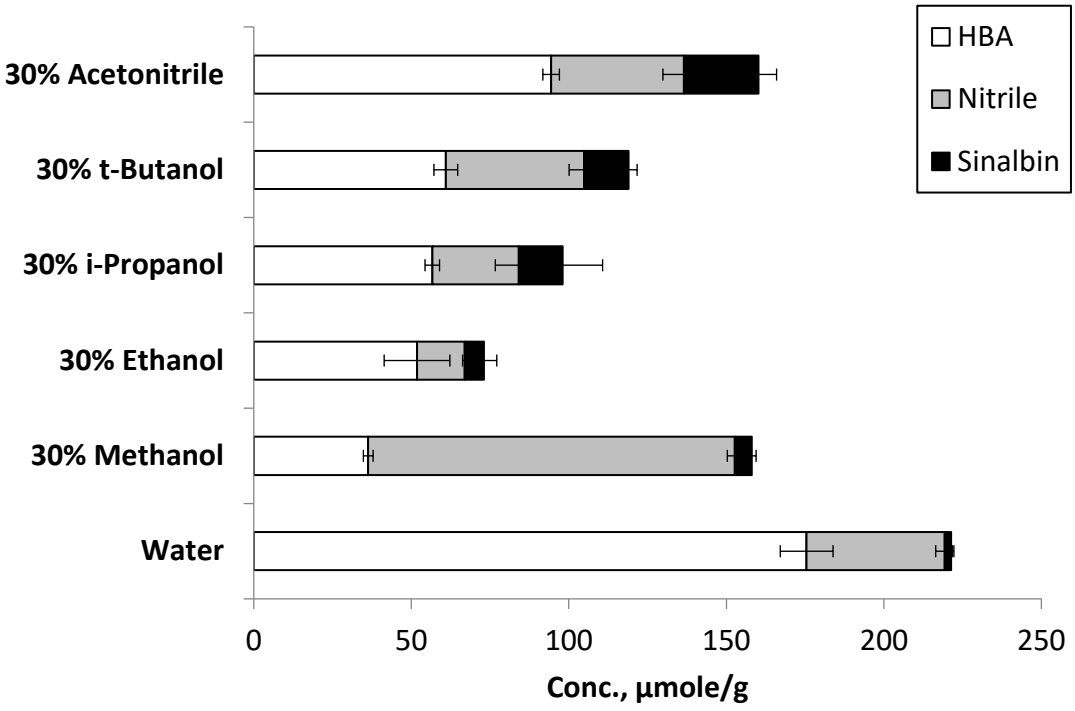

Figure 2.7. Extraction recoveries for intact sinalbin and its hydrolysis products from a series of 30% aqueous alcohol solutions. Horizontal bars represent standard deviations of the mean of three replicates.

For example, 4-hydroxybenzyl alcohol production was almost twice as high when *t*-butanol was used compared to methanol. Apparently, stabilization of 4-hydroxybenzyl isothiocyanate is reduced and the hydrolytic reaction proceeds to completion. Interestingly, acetonitrile also favored the formation of 4-hydroxybenzyl alcohol. However, due to the higher cost and toxicity of acetonitrile compared to water, its application for 4-hydroxybenzyl alcohol is not practical. Water was therefore selected as the most appropriate extractant because the addition of methanol is not required for HBA extraction and may promote the formation of unwanted reaction products.

Extraction of 4-hydroxybenzyl alcohol from Sinapis alba

Optimization of pH

To aid in the hydrolysis of sinalbin, pH of the extraction solution was optimized between 3.0 and 7.4, as this pH range represents that most favorable for myrosinase activity (Fig. 8). While complete hydrolysis of sinalbin was observed within the entire studied pH range, the production of HBA varied. Concentrations of HBA produced were similar from a pH value between 3.0 to 5.4, however at a pH greater than 3.8 incomplete hydrolysis was observed with a significant amount of sinalbin converted only to 4-hydroxybenzyl isothiocyanate. When the pH was further increased, a steady decline in HBA concentration was observed at the rate of approximately 34 µmol for each pH unit (Fig. 8). Such a decline can be explained by an increase in stability of the intermediate 4-hydroxybenzyl isothiocyanate as well as lower stability of HBA under basic conditions (Borek and Morra, 2005). While the selection of pH is critical for quantitative production of HBA, it also can affect the stability of HBA and consequently, shelf-life of any possible commercial product. The optimum pH was thus 3.8

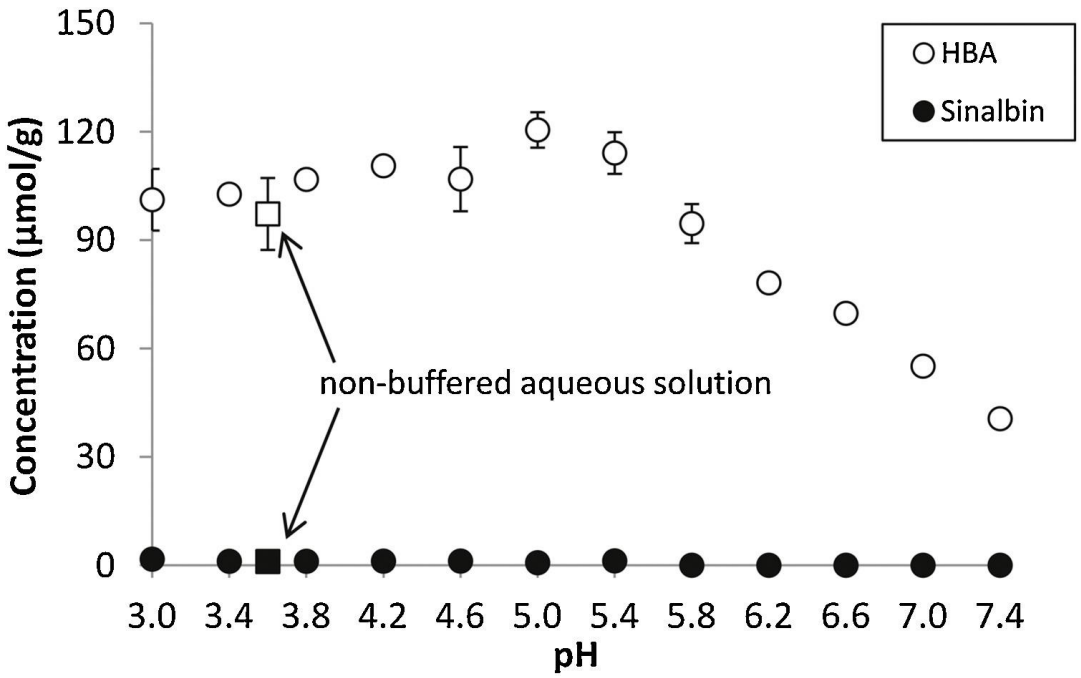

Figure 2.8. Sinalbin hydrolysis in aqueous solution at different pHs. Data points depicted with squares correspond to a non-buffered aqueous solution. Vertical bars represent standard deviations of the mean of three replicates.

as it resulted in complete hydrolysis of sinalbin with HBA being the predominant hydrolysis product.

Extraction time

To assure quantitative hydrolysis of sinalbin, time needed for complete hydrolysis was evaluated (Fig. 9). Under static conditions, production of HBA from mustard meal is limited by kinetics of sinalbin hydrolysis as controlled by enzymatic hydrolysis of sinalbin and non-enzymatic conversion of 4-OH benzyl isothiocyanate to HBA. Based on concentrations of HBA produced in aqueous solution, hydrolysis was more than 62% complete in 1 h, reaching 100% completion in 24 h. Incubation times longer than 24 h did not increase HBA recoveries, only serving to promote microbial growth resulting from protein and sugar availability in the extract. A hydrolysis time of 24-h was selected as optimal.

The optimized procedure to produce an *S. alba* mustard seed meal extract with an HBA concentration of 2 mM was as follows: ground mustard meal (0.1 g) was extracted with 5 mL 100 mM citrate-phosphate buffer (pH 3.8) in a 15-mL test tube using a Glas-Col Rugged Rotator (Glas-Col, Terre Haute, IN, USA) set at 60 rpm for 24 h. A higher concentration of HBA was achieved by increasing the meal to solvent ratio, however using less solvent resulted in lower recoveries. Specifically, when a 1:5 meal to solvent ratio was used instead of a 1:50 ratio, concentrations of HBA were increased by only two-fold (from 1.8 mM to 3.7 mM) as opposed to an expected ten-fold increase.

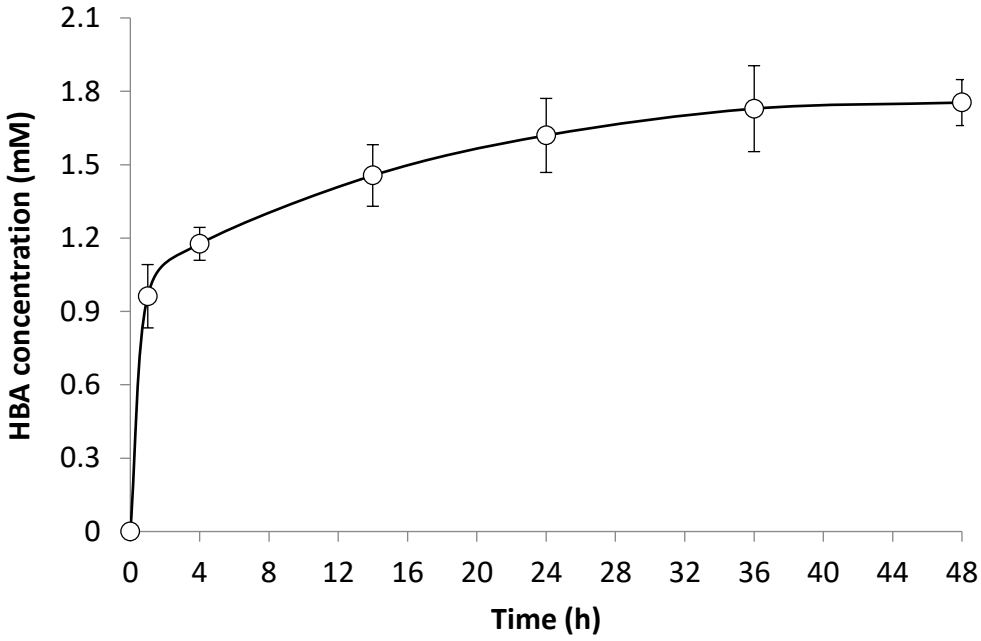

Figure 2.9. Concentration of produced HBA under the optimum hydrolysis and extraction conditions as a function of time. Vertical bars represent standard deviations of the mean of three replicates.

Extraction ionic thiocyanate from Sinapis alba

Extraction of ionic thiocyanate can be performed as a two-step process where sinalbin is extracted first, and then sinalbin is hydrolyzed by addition of myrosinase source (Fig. 10). Alternatively, ionic thiocyanate can be extracted in a one step process where hydrolysis and extraction combined. Both approaches can be used depending on the application goal and available equipment.

Approach 1

A two-step extraction approach is favorable in several scenarios. Extraction of sinalbin with alcohol prior to hydrolysis provides an extract that can be stored unrefrigerated for several weeks. This allows for a processing of large amounts of mustard meal at one time, reducing the amount of feedstock and reducing required storage space. Another scenario is in applications where co-extraction of antioxidants is desired along with ionic thiocyanate (Dubie et al., 2013). *Sinapis alba* contains sinapine, choline, snapic acid, and tannins among the compounds described as antioxidants. When co-extracted with sinalbin, they provide additional antimicrobial, antioxidant, and preservative properties to the extract that can be beneficial for some applications.

In the first step, conditions favoring myrosinase inhibition and sinalbin solubility are selected for the optimization of sinalbin extraction. Extraction of mustard meal carried out in 70-85% aqueous methanol or ethanol results in the highest concentration of sinalbin in the final extract (Fig. 5). After extraction, the extract is concentrated by rotary evaporation to achieve a syrup-like concentrate that can be freeze-dried to form hygroscopic amorphous solids. Sinalbin extract is stable in this form for more than a year, providing a more concentrated feedstock for ionic thiocyanate production as compared to mustard meal.

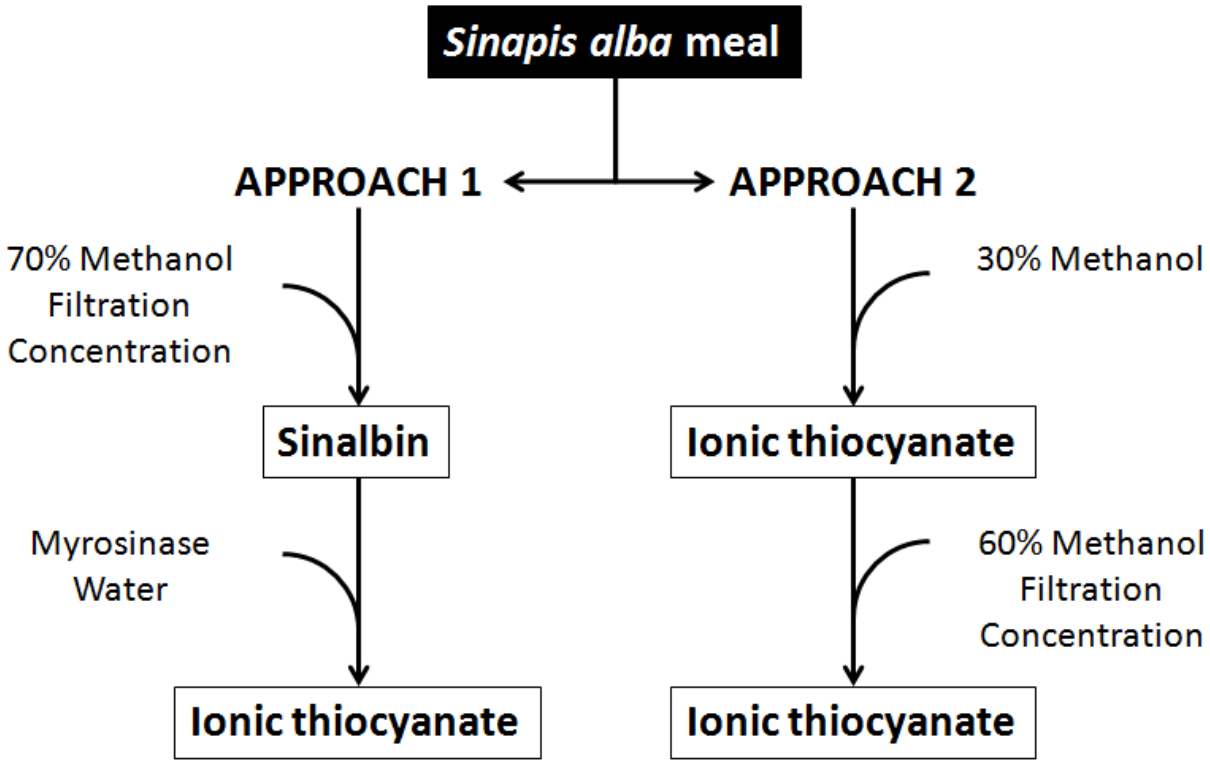

Figure 2.10. Scheme of two approaches for ionic thiocyanate extraction from *Sinapis alba*.

In the second step, a source of myrosinase is added to the sinalbin extract to facilitate hydrolysis. While myrosinase can be purified or obtained commercially, mustard meal can serve as an affordable myrosinase source. However, care should be taken to assure that myrosinase is active in the mustard meal used. Another consideration is the ratio of mustard meal to the amount of extract hydrolyzed relative to the total volume of hydrolysate (Fig. 11). It has been estimated that yellow mustard meal can swell to 400% of its original volume (Popova et al., 2017). As a result, recovery of ionic thiocyanate may be incomplete due to its sorption and precipitation on meal surfaces. It was estimated that up to 46% of ionic thiocyanate can be precipitated on the surface of mustard meal when the ratio of solvent to meal is less than 10 (Popova, unpublished data).

Similar to hydrolysis of sinalbin from mustard meal, hydrolysis of sinalbin in the extract is limited by pH of the medium, with an optimal pH between 5.0-6.0 (Popova et al., 2017). Unlike hydrolysis for production of HBA (Fig. 8), higher pH values do not necessarily lead to a decline in ionic thiocyanate concentration. However, dynamics of the hydrolysis reaction are altered, with the lower initial reactions rates and an increase in the reactions rates with lower pH as the reaction proceeds (Fig. 12). When the hydrolysis solution was buffered at pH 8.5, it took 25 times longer for the reaction to go to completion as compared to buffering at pH 6.5. While this may not be optimal for ionic thiocyanate production, it may be useful for designing slow release systems where gradual release during a specific time is required. One such purpose is field application of an extract-meal-buffer mixture where hydrolysis and release of ionic thiocyanate is activated by irrigation. Since ionic thiocyanate has been demonstrated to leach freely through the soil profile along with irrigation

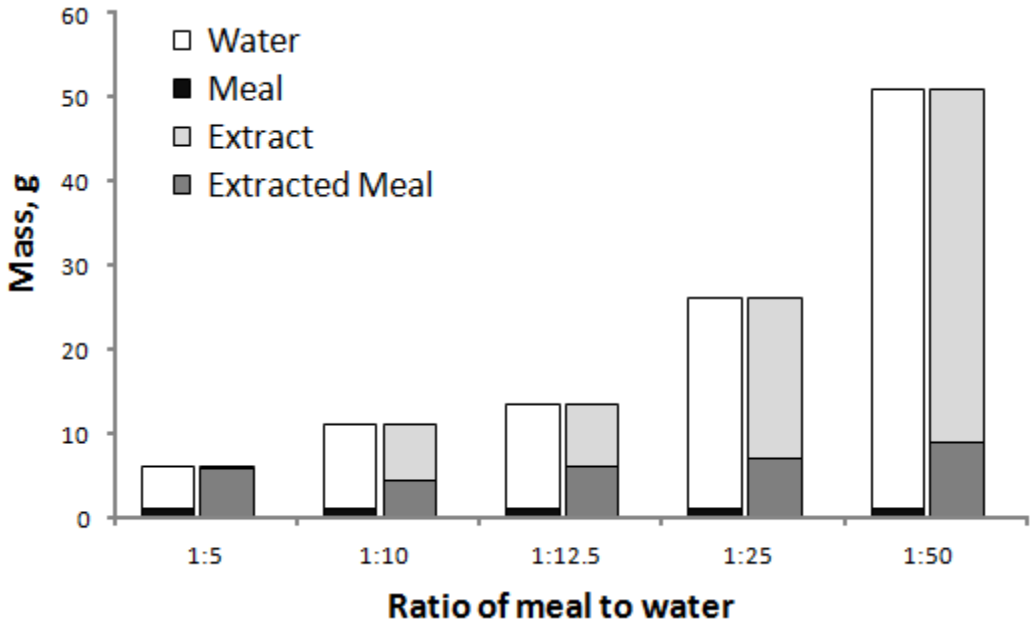

Figure 2.11. Relative proportions of water retention by *Sinapis alba* during aqueous extraction.

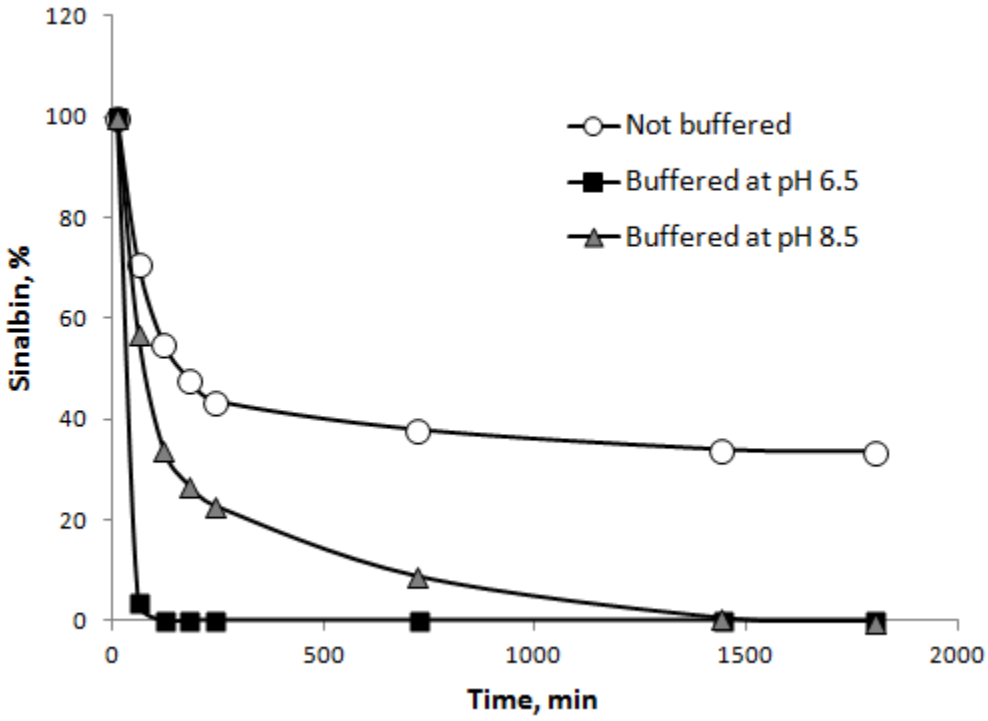

Figure 2.12. Hydrolysis of sinalbin from the mustard meal extract in the presence of mustard meal at the different initial pH.

water (Brown and Morra, 1993), one-time application may not be the best practice as most of the ionic thiocyanate will be lost.

Approach 2

The second approach for extraction of ionic thiocyanate is based on the hydrolysis of sinalbin followed by extraction of ionic thiocyanate (Fig. 10). The approach is somewhat different to approach 1 in the requirement for the use of an aqueous alcohol such as methanol or ethanol. Hydrolysis of sinalbin from mustard meal is carried out in 30% aqueous methanol to prevent microbial growth while still maintaining reasonable myrosinase activity. The second step, addition of methanol to the final concentration of 60%, is used to increase extractability of ionic thiocyanate, facilitate mucilage collapse, and improve filtration characteristics of the extract. As was mentioned previously, mucilage can swell significantly in aqueous media. Simultaneously, as the percentage of alcohol is increased, mucilage swelling is limited by the amount of free water molecules as they became associated with methanol through hydrogen bonding (Fig. 13). Thus, increasing the percentage of methanol in the extraction mixture leads to clearer extracts that are easy to filter. In addition, when water is used for extraction, cell walls in mustard meal are not complete disrupted and the transfer of glucosinolates to the bulk solvent is limited (Wang et al., 2010). Ionic thiocyanate recovery is also improved with an increase in methanol percentage in the extract (Fig. 14). When mustard meal was extracted with 30% methanol, the extracted meal contained a significant amount of ionic thiocyanate sorbed on the surface of the meal. Due to mucilage expansion of, ionic thiocyanate became trapped within the network of hydrocolloids. When

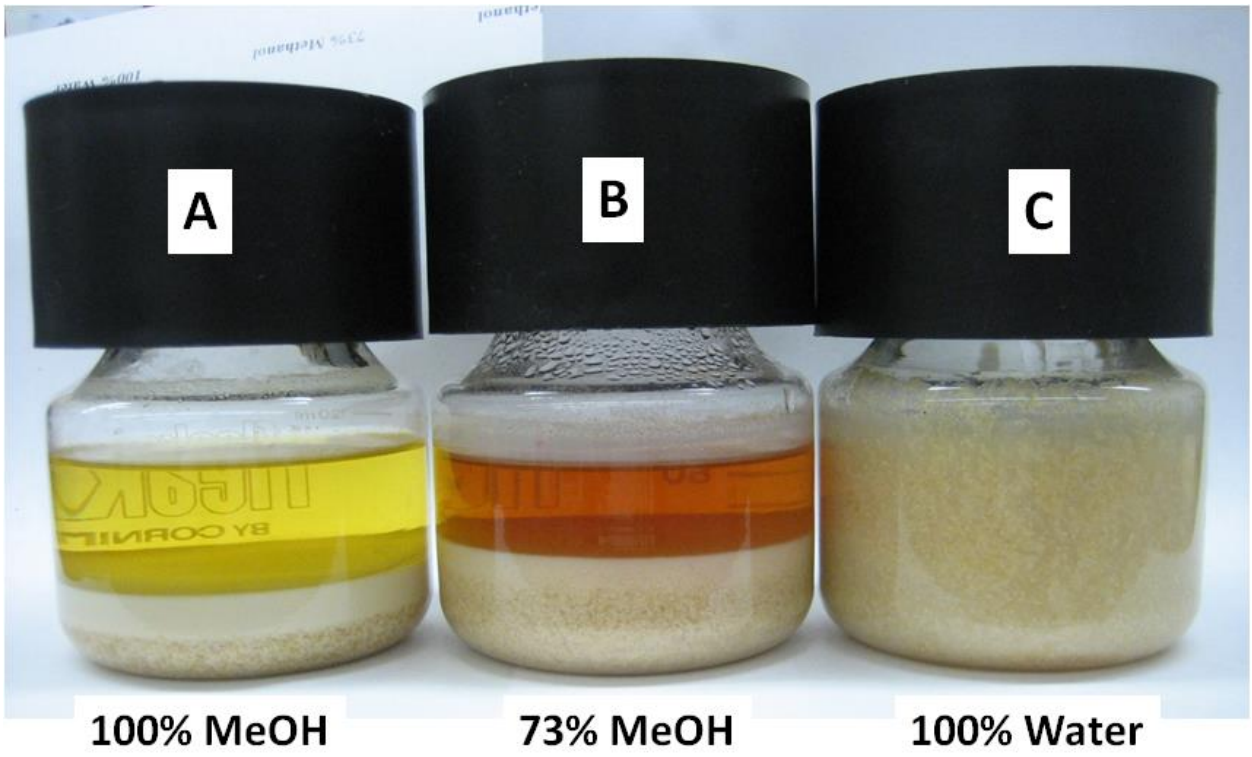

Figure 2.13. Swelling of *S. alba* meal (20 g) in aqueous methanol or water (100 mL) after 24 h.

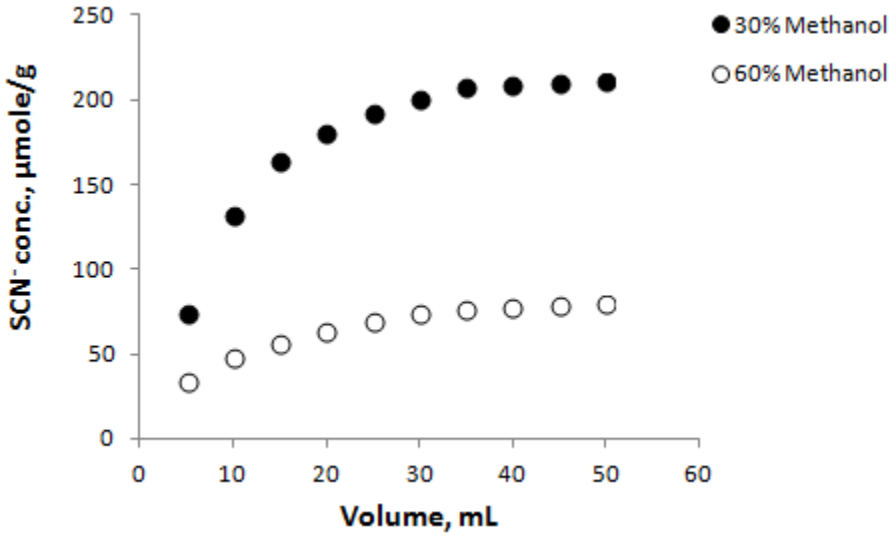

Figure 2.14. Recovery of ionic thiocyanate from *Sinapis alba* meal (1 g) extracted with 30% and 60% methanol (20 mL) by washing with 5-55 mL of water.

meal extracted with 30 or 60% methanol was washed, ionic thiocyanate recovered was three times higher when a lower methanol percentage was used.

Extraction of sinigrin from Brassica juncea

Extraction of sinigrin from *Brassica juncea* mustard meal can be performed using aqueous alcohols with concentrations higher than 60%, but preferably higher than 73% (Fig. 15). Chemical or physical pretreatment of the meal can be used prior to the extraction to deactivate myrosinase. For example, treatment of mustard meal with hexane can reduce myrosinase activity to allow for sinalbin extraction with a methanol percentage as low as 10% (Fig. 15). Similarly, autoclaving mustard meal prior to extraction can inactivate myrosinase (Eylen et al., 2006; Jen et al., 2001). However, when autoclaving is used, careful optimization of mustard meal moisture content and autoclaving temperature and time should be performed. In the present study, the best conditions for deactivation of myrosinase in *B. juncea* meal was achieved when meal was thinly spread and autoclaved at 115 °C for 15 min. Mustard meal moisture was adjusted to 10-20% by weight to facilitate heat transfer and thus prevent hydrolysis.

Hydrolysis of sinigrin extract

Hydrolysis of sinigrin from *B. juncea* meal results in the formation of 2-propenyl isothiocyanate (Popova et al., 2017). Since 2-propenyl isothiocyanate is volatile, hydrolysis of sinigrin needs to be carried out at the site and time of isothiocyanate application. For example, if 2-propenyl isothiocyanate is intended to be applied as a soil fumigant, hydrolysis needs to be initiated at the soil interface. This can be accomplished by applying sinigrin

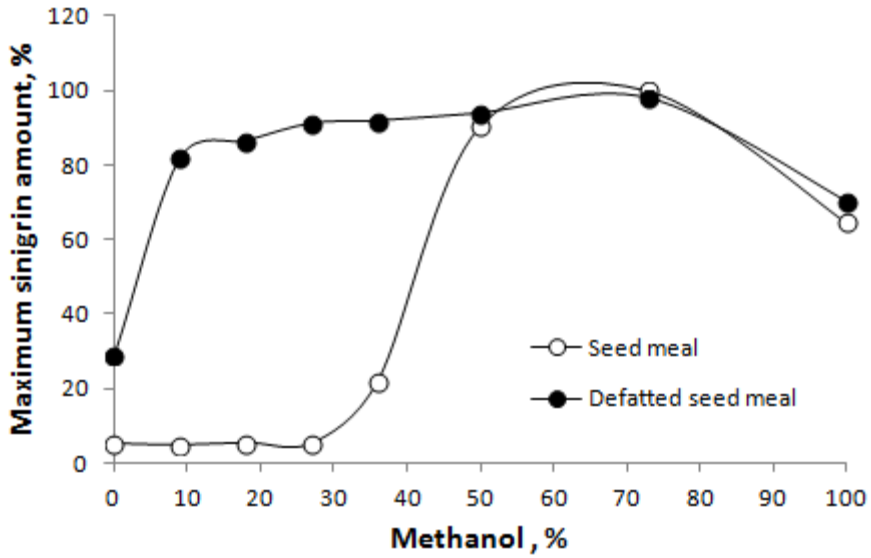

Figure 2.15. Extraction of sinigrin from *Brassica juncea* mustard meal before and after deffating with hexanes.

extract mixed with mustard meal as a source of myrosinase. Alternatively, 2-propenyl isothiocyanate can be produced with so-called fogging machines, where enzymatic hydrolysis is carried out in a liquid medium and volatile 2-propenyl isothiocyanate is used to fumigate the target medium. For example, 2-propenyl isothiocyanate generated in the field can be directly injected into soil. However, unlike hydrolysis of sinalbin that can be carried out off site, on site hydrolysis of sinigrin is more challenging to optimize as many variables must be optimized. Soil type, soil moisture, temperature, and relative humidity are among the factors that will affect sinigrin hydrolysis.

Drying of mustard meal extracts

Extracts produced from mustard meal can be dried to increase glucosinolate concentrations and to prolong the shelf-life of the active ingredient by reducing the content of water. For water-based extracts of *Sinapis alba*, several drying technologies such as be freeze-drying, spray-drying, drum-drying, or air-drying can be used (Fig. 16). The choice of drying technology is based on characteristics of the specific liquid extract as well as the desired properties of the final dry material. For example, when *S. alba* extract was drum- or air-dried, dense pliable flakes of material were formed (Fig. 16). These flakes did not dissolve in water rapidly and agitation was required to re-dissolve them in water. In contrast, freeze-dried and spray-dried material produced a free-flowing powder that was easily dissolved in the water. However, the bulk density of the produced material was lower as compared to drum-drying. Bulk density of the final product is important from the perspective of cost associated with shipping and compatibility with application equipment.

Figure 2.16. 30% Aqueous methanol extracts of *Sinapis alba* dried with different technologies: drum-drying, freeze-drying, and spray-drying.

For methanolic extracts, evaporation of organic solvent is required prior to drying. Evaporation must be carried out with care as extensive heating can result in thermal degradation of glucosinolates (MacLeod et al., 1981). After methanol was eliminated from 73% methanolic *Brassica juncea* extracts, they were air-dried and freeze-dried. Due to the large concentration of polysaccharides co-extracted with sinigrin, spray-drying was not possible. Polysaccharides have relatively low glass transition temperatures and, as a result, droplets of extract create wall depositions on the flying chamber rather than forming dry particles (Keshani et al., 2015). For the same reason, freeze-drying was challenging because an amorphous substance rather than a dry product was occasionally produced with no clear correlation to the drying conditions or extract characteristics. Air-drying produced a crystalline material. Both freeze-dried and air-dried material were extremely hygroscopic and required desiccated conditions for storage.

Concentration of glucosinolates in dried extracts was not noticeably affected by the drying technique as long as moisture content was taken into consideration. For instance, concentration of ionic thiocyanate and 4-hydroxybenzyl alcohol in 30% methanolic extracts were similar in spray- and freeze-dried extracts (Fig. 17). Similar trends were observed for *Brassica juncea* extract produced by air- and freeze-drying.

Concluding remarks

The present research demonstrates that mustard seed meal can be extracted for production as vital alternatives to conventional pesticides. It is possible to produce a powder extracts that contain ionic thiocyanate, 4-hydroxybenzyl alcohol, or an inactive form of 2-propenyl as the active ingredient. The powder may be reconstituted in water and applied as a spray or incorporated in soil as a pre- or post-emerge amendment to be used for control of a wide

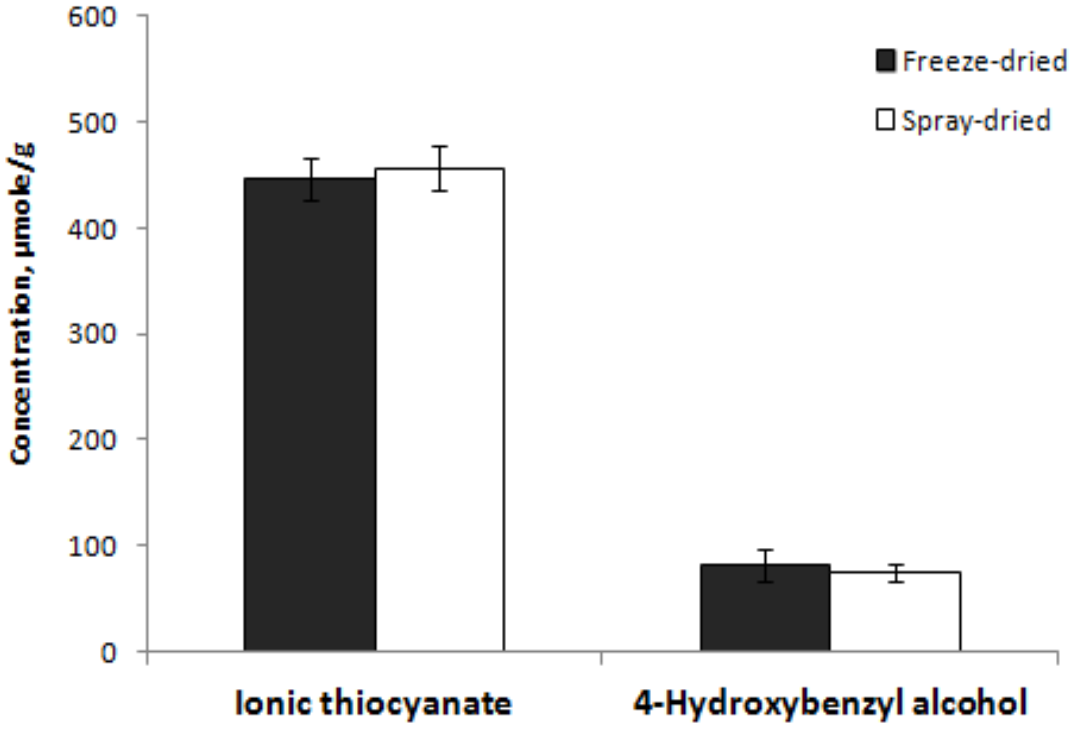

Figure 2.17. Concentration of herbicidal ingredients in freeze- or spray-dried extracts of 30% methanolic extracts of *Sinapis alba*.

range of pest. However, field trial is still needed to test extracts on crops grown under real-world conditions. The formulated extracts can potentially be prepared and formulated so that they will be suitable for organic certification.

Chapter 3: *Solanum Sisymbriifolium* Glycoalkaloids as Natural Pesticides

Introduction

Properties and occurrence of glycoalkaloids

Glycoalkaloids represent a wide class of chemical compounds composed of steroidal alkaloid and sugar moieties. In plants, steroidal glycoalkaloid aglycones are synthesized from cholesterol as a precursor by incorporating an amine group through side chain modification to form spirosolane- and solanidine-type aglycones (Ginzberg at al., 2009; Itkin et al., 2013) (Fig. 18). These two types of aglycones are further then modified under the action of oxidoreductases (Friedman, 2006). Finally, glycosyltransferase enzyme catalyzes glycosylation of the aglycone to form a series of glycoalkaloids (Friedman, 2006). Steroidal glycoalkaloid consist of a C27 cholestane skeleton to which a carbohydrate moiety of one to five monosaccharides is attached (van Gelder, 1991; Roddick, 1996). The steroidal glycoalkaloids are found in more than 350 plant species, mainly of *Solanaceae* family such as tomato, potato, pepepr, and aubergines (Roddick and Melchers, 1985). Glycoalkaloids are present in all plant organs with the concentration varying depending on the plant growth stage, environmental conditions, and specific plant species. For example, concentrations of glycoalkaloids in potato are highest in sprouts (2,750-10,000 mg kg^{-1}) (Friedman and Dao, 1992). When the plant initiates growth, glycoalkaloids accumulate in stems (320-450 mg kg^{-1}), leaves (1450 mg kg^{-1}), berries (380-860 mg kg^{-1}), and roots (860 mg kg^{-1}) (Friedman and Dao, 1992). The concentration of glycoalkaloids in tubers is low (18-150 mg kg^{-1}), with compounds predominantly concentrated in peels (850 mg kg^{-1}) (Friedman and Dao, 1992). In non-tuber plants, such as tomato, similar distribution of glycoalkaloids is observed. Concentrations of glycoalkaloids can vary significantly due to environmental

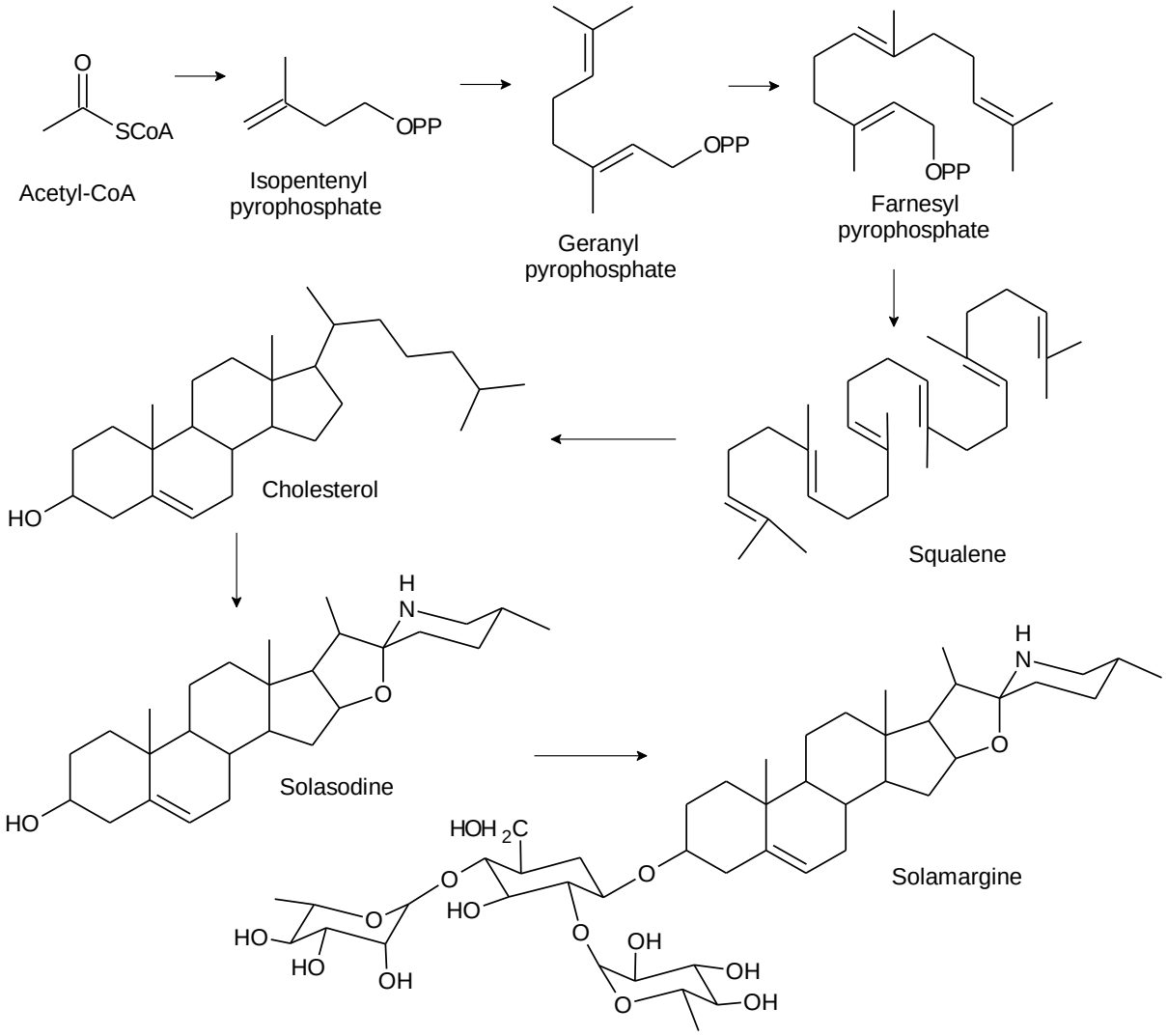

Figure 3.1. Glycoalkaloid biosynthesis pathway.

conditions. For example, when potato plants were exposed to drought, the concentration of glycoalkaloids increased by 2-79% depending on the cultivar, with concentrations of glycoalkaloids increased up to 250% in the flesh of potato tubers (Bejarano et al., 2000).

Pesticidal properties of glycoalkaloids

While glycoalkaloids are not considered to be essential from plant growth, they have been linked to plant resistance to pests and pathogens. Plants rich in glycoalkaloids have been shown to have resistance to a variety of pests (Table 2). For example, wild species of potato are resistant to Colorado beetles (*Leptinotarsa decemlineata*) (Schreiber, 1957; Baranova et al., 1952; Tingley, 1984). Plant foliage of tomato plants containing α-tomatine was shown to reduce feeding and increase mortality of Colorado beetles as well (Flanders et al., 1992). Extracts from potato containing α-chaconine were efficient against other two beetles *Tenebrio molitor* and *Zophobas atratus* (Wierenga and Hollingworth,1992). Tomatine was shown to induce mortality of potato leafhopper Empoasca fabae (Sanford et al., 1996). Glucosinolates from two wild potato species were resistant against potato leafhopper *Empoasca fabae* (Tingey et al., 1978; Weissenberg et al., 1998). Extracts from several *Solanum* plants had inhibitory activity toward the growth of *Tribolium catenum* and *Manduca sexta* larvae (Weissenberg et al., 1998). Glycoalkaloids also exhibit molluscicidal properties toward *Helix aspersa, Lymnea cubensis, Biomphalaria glabrata, Bulinus truncatus* (Smith et al., 2001; Alzerreca and Hart, 1982; Bekkouche et al., 2000).

Pesticidal properties of glycoalkaloids are not widely studied and the exact mechanism of action for pesticidal activity is not known. However, it has been demonstrated

Table 3.1. Pesticidal properties of glycoalkaloids toward pests.

Pest controlled	Plant species	Glykoalkaloids	Reference
Biomphalaria glabrata	*Solanum mammosum*	α-Solasonine, α-Solamargine	Alzerreca and Hart, 1982
Bulinus truncatus	*Solanum sodomaeum*	not reported	Bekkouche et al., 2000
Bulinus truncatus	*Solanum elaegnifolium*	not reported	Bekkouche et al., 2000
Empoasca fabae	*Solanum polyadenium*	α-Chaconine, α-Solanine	Tingey et al., 1978
Empoasca fabae	*Solanum bulbocastanum*	α-Chaconine, α-Solanine	Tingey et al., 1978
Empoasca fabae	*Solanum berthaultii*	α-Solasonine, α-Solamargine	Sanford et al., 1996
Empoasca fabae	*Solanum chacoense*	Leptine I, Leptine II	Sanford et al., 1996
Empoasca fabae	*Solanum tuberosum*	Chaconine	Sanford et al., 1996
Empoasca fabae	*Solanum lycopersicum*	Tomatine	Sanford et al., 1996
Helix aspersa	*Solanum tuberosum*	α-Chaconine, α-Solanine	Smith et al, 2001
Leptinotarsa decemlineata	*Solanum tuberosum*	Leptine, α-Chaconine	Schreiber, 1957; Baranova et al., 1952; Tingley, 1984, Wierenga and Hollingworth,1992
Leptinotarsa decemlineata	*Solanum lycopersicum*	α-Tomatine	Flanders et al., 1992
Lymnea cubensis	*Solanum mammosum*	α-Solasonine, α-Solamargine	Alzerreca and Hart, 1982
Manduca sexta	*Solanum dulcamara*	α-Tomatine	Weissenberg et al., 1998
Tenebrio molitor	*Solanum tuberosum*	α-Chaconine	Wierenga and Hollingworth,1992
Tribolium castaneum	*Solanum khasianum*	Solamargine, Solasonine, α-Tomatinol	Weissenberg et al., 1998
Zophobas atratus	*Solanum tuberosum*	α-Chaconine	Wierenga and Hollingworth,1992

that glycoalkaloids have two major mechanisms of biological action: 1) inhibition of cholinesterases enzymes and 2) membrane disruption (Milner et al., 2011).

Acetyl- and butyrylcholinesterase are enzymes that catalyzes hydrolysis of neurotransmitter cholines - acetylcholine and butyrylcholine, respectively. Inhibition of acetyl- and butyrylcholinesterase results in accumulation of acetylcholine in the synaptic cleft and, as a result, disruption of neurotransmission (Milner et al., 2011). It is believed that the inhibition is due to a competitive non-covalent binding to the enzyme's active sites (Wierenga and Hollingworth,1992; Nigg et al., 1996). Apparently, the presence of the sugar moiety is required for binding as the aglycone has no effect on cholinesterases activities and the amount of sugar moieties was directly proportional to the inhibition effect (Wierenga and Hollingworth,1992). At the same time, aglycone type has a significant effect on the inhibition effect. For example, both α-solamargine and α-solasonine have a chacotriose sugar moiety, however, inhibitory activity of α-solasonine was at least 10 times lower (Roddick 1989). Still, the concentrations of glycoalkaloids needed for cholinesterases inhibition are rather high with up to 10 mM of α-chaconine or α-solanine for inhibition of acetylcholinesterase (Friedman, 2006).

Glycoalkaloids of Solanum Sisymbriifolium

Since *Solanum sisymbriifolium* does not represent agronomical value per se, chemistry of this plant is poorly studied (More 2019; Pasdaran et al., 2017). While several potentially biologically active compounds were described, only five glycoalkaloids were identified in *S. sisymbriifolium* (Table 3). The chemical composition of *S. sisymbriifolium*

Table 3.2. Glycoalkaloids identified in *Solanum sisymbriifolium*.

Glycoalkaloid	Aglycone	Sugar moieties	Plant part	Reference
Nuatigenosido	Nuatigenin	Chacotriose	Roots	Ibarrola et al., 2006
Solamargine, β-Solamarine	Solasonine	Chacotriose Solatriose	Fruit	Bagalwa et al., 2010
Isonuatigenin-3-O-β-solatriose	Isonuatigenin	Solatriose	Roots	Ferro et al., 2005
Nuatigenin-3-O-β-chacotriose	Nuatigenin	Chacotriose	Roots	Ibarrola et al., 2011
Glycoalkaloids were hydrolyzed prior to the extraction	Solasodine	Not reported	Fruits	Chauhan et al., 2011

has been historically studied in regards to its use in traditional medicine by indigenous people of Central and South America (More 2019). Intact glycoalkaloids are generally credited with anticancer activities while glycoalkaloid aglycones are credited with contraceptive properties (More 2019). Solamargine and solamarine are the two most commonly cited glycoalkaloids found in *S. sisymbriifolium*. Both glycoalkaloids have the same aglycone solasonine, but different sugar moieties (Friedman 2006). These glycoalkaloids were first isolated from fruits, but later detected in all parts of *S. sisymbriifolium* plant (Bagalwa et al., 2010). Recently several reports were published regarding the identification of three new glycoalkaloids from root extracts of *S. sisymbriifolium* (Ibarrola et al., 2006; Ferro et al., 2005; Ibarrola et al., 2011). Glycoalkaloids with nuatigenin and isonuatigenin aglycone were isolated from plant roots to be tested for hypertensive activity (Ferro et al., 2005).

Materials and Methods

Plant material preparation

Plants were collected from greenhouse at 6-week age. Plant were cleaned from loose soil, fresh frozen in liquid nitrogen, and freeze-dried for 72 h. After freeze-drying, roots, stems, leaves, and buds/flowers were separated, pulverized to 1 µm with Cyclon sample mill (UDY Corporation, Fort Collins, CO, USA), and kept at in –20 °C freezer until analysis.

Glycoalkaloid extraction and fractionation

Extraction of glycoalkaloids was performed by refluxing of 1 g plant material (flowers or roots) with 100 mL of 90% aqueous methanol for 4 h. After extraction, the slurry was

vacuum filtered through Buchner funnel lined with Whatman filter #42. Extraction was repeated two more times, filtrates were combined, and evaporated under vacuum to 50 mL. Concentrated methanolic extract was extracted twice with 50 mL of hexanes to remove chlorophyll and lipids. After separation by centrifugation (1000 g for 2 min), methanolic extract was evaporated until dryness by rotatory evaporation. Dry residues were resuspended in 50 mL of water saturated *n*-butanol to remove water soluble pigments. Organic fraction was separated by centrifugation (1000 g for 2 min), evaporated to dryness and dry residues were resuspended in 2 mL of methanol. Methanol was diluted with 200 mL of DI water and subjected to SPE fractionation.

SPE was carried on Waters Oasis HLB cartridges that were preconditioned with 6 mL of methanol and 6 mL of water. After extract was loaded under 10 mm Hg negative pressure, cartridges were dried for 10 min, and washed with 20% aqueous methanol. Glycoalkaloids were eluted with 6 mL of 22-88% aqueous methanol in 3 % increments. Total of 23 fractions was collected. Fractions were collected and kept in –20 °C freezer until analysis by HPLC/ TOF MS.

Glycoalkaloid hydrolysis

Glycoalkaloids were hydrolyzed by refluxing plant material (1 g) with 20 mL of 10% aqueous methanol containing 1 mL of concentrated HCl for 1, 2, or 4 h. After centrifugation at 4000 g supernatant was decanted, neutralized with NaOH, and concentrated under vacuum to 2 mL. Hydrolysate was diluted with 200 mL of DI water and subjected to SPE purification.

HPLC/ TOF detection of glycoalkaloids

HPLC analysis was performed using an Agilent 1200 Series HPLC with a diode array detector (DAD) coupled to an Agilent G6230 TOF MS equipped with an ESI source (Agilent, Santa Clara, CA, USA). The chromatographic separation of glycoalkaloids was performed on an Extend-C18 3.5um, 2.1x100 mm (Agilent Technologies Inc., Santa Clara, CA, USA) reversed phase chromatographic column. HPLC conditions were optimized for the separation of glycoalkaloids by adjusting following parameters: mobile phase composition, gradient steepness, flow rate, column temperature and injection volume. The optimized conditions were as followed: The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient program started with isocratic elution using 10% B for 2 min, followed by a linear gradient to 25% B from 2 to 5.5 min, followed by a linear gradient to 60% in 3.5 min, then organic solvent was increased to 90% B in 1 min, kept at 90% for 2 min, and re-equilibrated back to the initial mobile phase composition in 7 min. Column was maintained at 30 °C. The injection volume was 5 μL. The flow rate was 0.4 mL min^{-1} and spectra were recorded from 190 to 400 nm.

Both flow-dependent (nebulizer pressure, drying gas flow rate, and temperature) and flow-independent (electrospray ionization potential, and collision-induced dissociation potential) MS TOF parameters were optimized using a gradient elution HPLC program. Then using a syringe pump, electrolyte solution (ammonium formate, formic acid, or acetic acid) was introduced through a T-coupling into the ion source along with eluate from the HPLC. Such a setup allows for molecular ion identification without a change in HPLC retention time.

The final TOF MS condition were as follows: Electrospray ionization was operated in the positive mode. The absolute values for electrospray ionization potential and collision-induced dissociation potential were 3500 and 250 V, respectively. Gas temperature was 350 °C, drying gas (N_2) flow rate was 10 L min^{-1}, and nebulizer pressure was 2.4×10^5 Pa. The analyses were conducted in a centroid mode within an m/z range from 100 to 1700 amu.

HPLC/ TOF data analysis

HPLC TOF MS analyses were processed in MassHunter B.08.00 Agilent Profinder software (Agilent technologies Inc, Santa Clara, CA, USA) in positive mode, which shows better ionization and a larger number of peaks. *In silico* identification was carried out using a MS-DIAL software by integrating metabolome databases with mass spectrometry cheminformatics (Tsugawa et al., 2015). MS-DIAL is an open-source software for data independent based identification and quantification of small molecules by mass spectral deconvolution. Several other open source programs were used including CSI:FingerID (Duhrkop et al., 2015), SIRIUS (Duhrkop and Bocker, 2015), MINE database (Jeffryes et al., 2015), and Spektraris (Cuthbertson et al., 2013).

Acetylcholinesterase assay

Measurement of acetylcholinesterase (EC number 3.1.1.7) activity was conducted in 96-well microplate format according to a method previously described with some modifications (Ellman et al., 1961). Briefly, 100 µL of acetylcholinesterase from

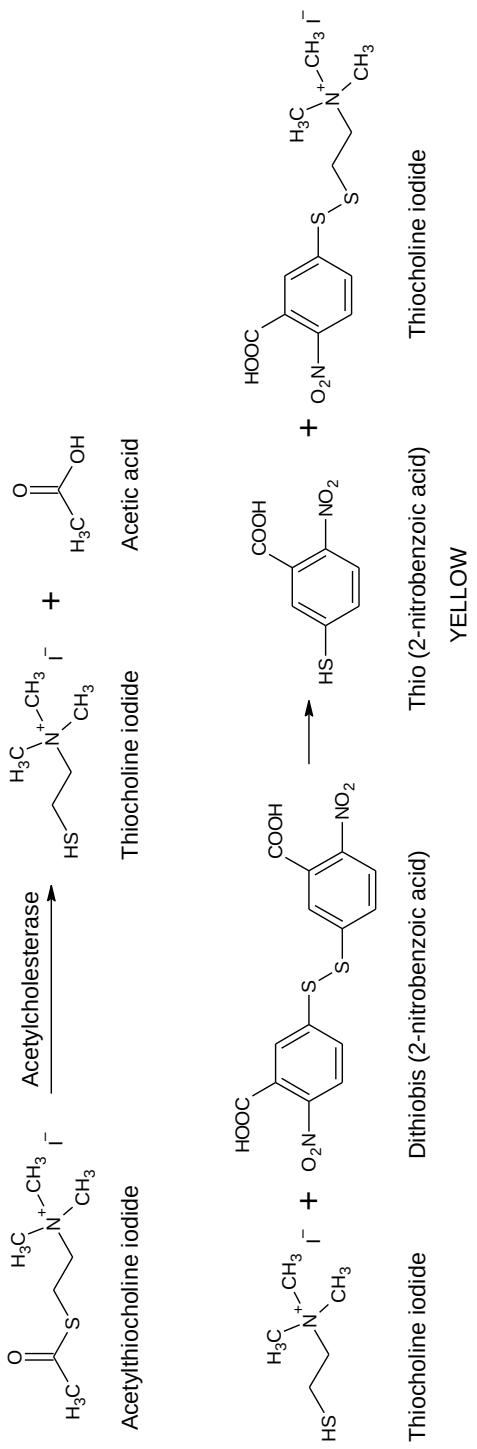

Figure 3.2. Scheme of Ellman reaction for quantifying acetylcholesterase activity.

Electrophorus electricus (1 units/mL) and 100 µL of modified Ellman's reagent (1 mM acetylthiocholine and 0.3 mM 5,5′-dithiobis (2-nitrobenzoic acid) in (100 mM pH 7.4 phosphate buffer) were added to 100 µL of phosphate buffer (100 mM, pH 7.4) containing 0.1, 1, 10, or 100 µL of plant extract reconstituted in water (Fig. 19). Once the reaction was started, the absorbance at 405 nm was measured every minute for 1 h using SpectraMax M Series Multi-Mode Microplate Readers (Molecular Devices, LLC., San Jose, CA, USA). The rate of the reaction was calculated as a slope of the kinetics curve over the linear range (30 min for most of the kinetic curves). Inhibition was calculated as a percentage difference between the control (with no plant extract present) and the specific sample.

Results and Discussions

HPLC optimization for detection of glycoalkaloids

Optimization of HPLC operating conditions was done using a representative extract from *S. sisymbriifolium* flowers. While the preferable method for condition optimization would be to use corresponding analytical standards, it was not possible due to the lack of available standards. To the best of our knowledge, only solamargine is commercially available; while other *S. sisymbriifolium* glycoalkaloids are either not available or not identified. Also, since *S. sisymbriifolium* chemistry is poorly studied, the exact number and identity of glycoalkaloids are unknown, thus presenting a challenge for HPLC separation. However, based on the studies of other plants rich in glycoalkaloids, it is known that isomeric forms of glycoalkaloids are often present including constitutional isomers, stereoisomers, diastereomers, and enantiomers. As a result, the several chromatographic peaks with exact monoisomeric mass can be present (Fig. 20). For example, four peaks

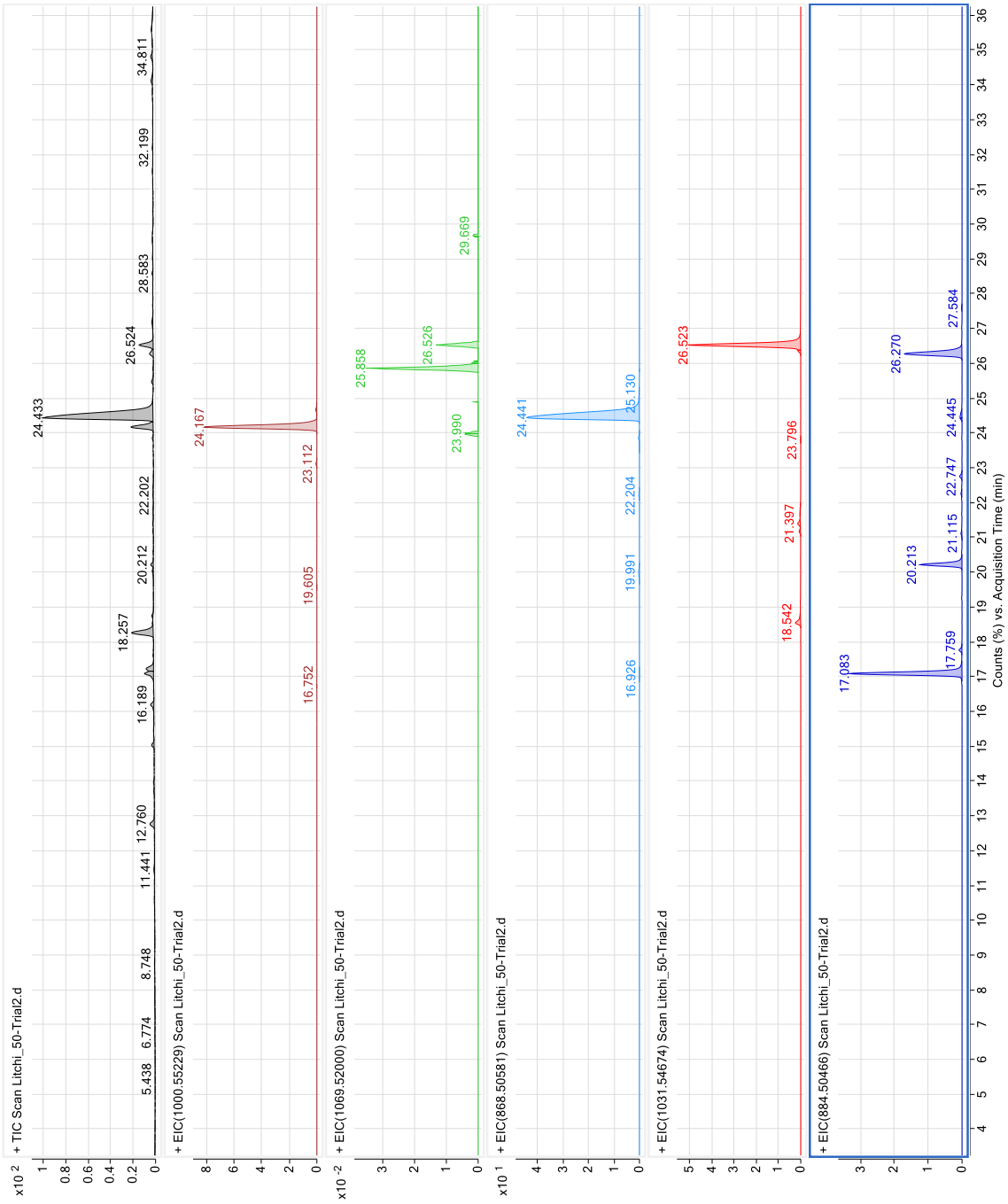

Figure 3.3. HPLC/TOF MS TIC chromatogram of flowers/buds *Solanum sisymbriifolium* methanolic extract.

with monoisotopic mass of 1069.5241 and four monoisotopic and three peaks with monoisotopic mass of 844.5068 were present.

To achieve the best separation of all glycoalkaloids, a very slow gradient was used. However, even with 0.9 % of acetonitrile/ min gradient steepness, no baseline separation was achieved as the peaks were clearly overlapped in the chromatogram with most of the glycoalkaloids eluting in 15 – 30 min interval (Fig. 20). While peaks for glycoalkaloids with higher abundance can be deconvoluted with mass spectroscopy software, the less abundant glycoalkaloids with poor expressed fragmentation patterns would be harder to analyze. Based on the long elution gradient, the five most abundant glycoalkaloids were identified (Fig. 20). For the analysis of these five compounds, a shortened elution method was developed (Table 4).

TOF MS optimization for detection of glycoalkaloids

Since the optimization for the TOF MS signal was performed on a plant sample rather than pure standards, a two-step approach was used. First, the MS parameters were optimized to achieve the highest MS response signal that allowed to confirm the presence of compound peaks. Once the peaks with high enough total ion current were obtained, the molecular ion was confirmed by using different ionization matrix modifier and analyzing samples in positive and negative ESI modes (Figure Suppl. 1). Upon electrospray ionization, along with the protonated (positive ESI) or deprotonated (negative ESI) molecules, a series of adducts with solvent, ammonium, or sodium are commonly observed (Kruve and Kaupmees, 2017). The formation of adducts is possible because the surface excess charge of ESI nanodroplets may be carried by cationic species as the case in the positive ESI. In addition, dimers or

Table 3.3. HPLC elution gradients for the separation of glycoalkaloids.

Program 1		Program 2	
Time, min	Acetonitrile, %	Time, min	Acetonitrile, %
2	5	2	10
35	35	5.5	25
45	98	9	60
50	98	10	90
		12	60

Analyte	Retention time, min	Retention time, min
Glycoalkaloid 1016	25.469	9.071
Glycoalkaloid 884	26.270	9.213
Glycoalkaloid 1031	26.523	9.132
Glycoalkaloid 1000	24.167	8.773
Glycoalkaloid 868	24.441	8.835

trimers of the molecule can be formed under the saturated conditions. Thus, it is not always appropriate to assign the molecular ion in the mass spectra based on the highest mass. This, in turn, creates a challenge for *de novo* and *in silico* identification as it is based on the correct assignment of the molecular ion.

Thus, to avoid erroneous data analysis, spectra for studied glycoalkaloids were obtained in acetonitrile-water and acetonitrile-water with the addition of formic acid, ammonium formate, and acetic acid. By using the different ionization matrix modifiers in the solvent system, production of different adducts is promoted. For example, in the absence of electrolyte or in the presence of formic acid, sodium adducts dominated in the positive ESI mode. Addition of ammonium formate, for example, facilitates formation of ammonium adducts. By comparing the difference between formed ions and correlating it with ammonia and sodium adducts, the molecular ion can be determined. Introduction of the ionization matrix modifier post column rather than by flow injection analysis was done to avoid changes in compounds' retention times due to the difference in mobile phase.

For all five major glycoalkaloids, molecular ions were identified based on the major with the largest m/z value that was detected regardless of the ionization matrix modifier used (Figure Suppl. 1). For example, for solamargine a molecular ion of 868.5089 was present positive ESI mode and 866.4828 was present in negative ESI mode. While in the positive mode, no apparent adducts were detected with all studied ionization matrix modifiers, several adducts were present in negative ESI mode. Specifically, 912.4872 ion that is an adduct with formic acid $[M+HCO_2H-H]^-$ and 926.5029 ion that is an adduct with acetic acid $[M+CH_3CO_2H-H]^-$. Interestingly, the other four glycoalkaloids were not as susceptible to adduct formation as solamargine. It is not clear if such a difference is due to the fact that the

peak area and intensity of solamargine is significantly higher or if it due to the difference in preferential ion formation among different glycoalkaloids. While both positive and negative ESI provided clear spectra, signal intensity in positive ESI was about 40 times higher.

Fragmentation patterns of the five major glycoalkaloids were studied using collision-induced dissociation potential between 75 V to 300 V (Figure Suppl. 1). The highest fragmentation occurs at 100, 125, and 150 V that can be explain in terms of relatively high voltage that promotes breakage of the molecule, but not high enough to interfere with the transport of ion to the capillary. At 200 V, fragmentation is reduced, and at 300 V, mostly molecular ions are present in the spectra. The total intensity of the ions was the highest at 250 V, and thus, 250 V was used for the following studies.

For solamargine, three major fragments were present in the mass spectra: 722.4532, 576.3938, and 414.3400. Fragments were assigned based on the previously published fragmentation pattern (Distl and Wink, 2009) and neutral loss mass analysis. The ion fragment of 414.3400 is an aglycone of solamargine, solasodine. The other two fragments correspond to the loss of one and two rhamnose moieties. The neutral loss of sugar moieties is one of the identification characteristics for glycoalkaloids spectra interpretation. While the aglycone can be different and is not necessarily known, the pattern of sugar moieties loss along with the molecular ion can be indicative of glycoalkaloids. Also, sugar moieties neutral loss can be used to distinguish among glycoalkaloids with the same molecular formula. For example, in *S. sisymbriifolium* extract glycoalkaloid with molecular ion of 884.5069 was present. The corresponding molecular formula for the compounds would be $C_{45}H_{73}NO_{16}$. Indeed, this molecular formula belongs to solasonine - compound previously reported in *S. sisymbriifolium* (Friedman, 2006). However, based on the sugar moiety loss pattern, the

compound present is not solasonine as the sugar moiety and aglycone are different. The aglycone was assigned as 430.3347 ion, which would most likely be hydroxylated solasodine. The sugar moiety loss is consistent with the sugar moiety loss in solamargine molecule: two rhamnose are lost consequently first, and glucose is lost last. Thus, the compound is more likely hydroxylated solamargine rather than solasonine. However, NMR analysis is needed to confirm or refute that. Indeed, compound in *Solanum nigrum* with m/z 884.5025 (calculated mass for $C_{45}H_{74}NO_{16}$, 884.5008) has been recently identified as 7α-OH solamargine (Gu et al., 2018). Also, an aglycone with m/z 430.3430 (calculated mass for $C_{27}H_{44}NO_3$, 430.3469) was detected in *Solanum surrattense* extract that was identified as 1β, 3β, dihydroxy, 22 αN-spirosol5-ene (Nawaz et al., 2014).

Similarly, a pair of compounds with molecular ions 1000.5547 and 1016.5500 can be tentatively identified as sycophantine and hydroxylated sycophantine, respectively. Both compounds have the same sugar moiety neutral loss pattern: xylose is lost first, then followed by the loss of two rhamnoses and glucose. This sequence of the sugar moieties is consistent with the reported previously structure of sycophantine. While sycophantine has not been previously reported in *S. sisymbriifolium* previously, the aglycone molecular weight was the same as for solamargine. Sycophantine and solamargine have the same aglycone, thus, the presence of sycophantine in *S. sisymbriifolium* is rather possible. The presence of hydroxylated sycophantine is also consistent with the hypothesis that both compounds are related to solamargine by the aglycone skeleton but have different sugar moiety. In fact, it is common to find several glycoalkaloids with the same aglycone but different sugar moieties in the same plant (Friedman, 2006). In fact, it was demonstrated that glycoalkaloids with

chacotriose side chain exhibit significantly higher nematocidal activity that glycoalkaloids comprised of the same aglycone but with solatriose sugar moiety (Udalova et al., 2004).

Solid phase extraction of Solanum Sisymbriifolium glycoalkaloids

Solid phase extraction (SPE) was used for purification as well as semi-fractionation of *Solanum sisymbriifolium* extract (Fig. 21). Both root and flower/bud extracts were purified by SPE. All five glycoalkaloids eluted from SPE cartridge in the range 2834 – 76% methanol. Elution order was consistent with the chromatographic elution order thus suggesting hydrophilic interactions are being the selecting factor. The difference in the specific distribution of the eluted amount between root and flower/bud extracts is likely due to the concentration difference. Total solamargine load in flower/bud extract was 217 µg and the total load in root extract was only 6.3 µg. Thus, the capacity of SPE cartridge was likely exceeded in the case of flower/bud extract and wide elution peaks for glycoalkaloids are observed. For example, in flower/bud extract, solamargine elutes in 12 fractions (from 40 to 73% methanol). In root extract, solamargine elutes 3 fractions (from 67 to 73% methanol). While the decrease in glycoalkaloid load make the elution peaks narrower, SPE still cannot completely resolve the mixture of glycoalkaloids present in the extracts. In root extract, all five glycoalkaloids overlap even thought their elution is limited to 3-4 fractions. This fact is also consistent with the HPLC results where glycoalkaloid peaks overlap even with very shallow elution solvent gradient. Thus, it would be incorrect to rely on SPE for purification of individual glycoalkaloids. However, SPE still can be used for the separation of glycoalkaloids as a group of compounds where elution is performed with 76% methanol.

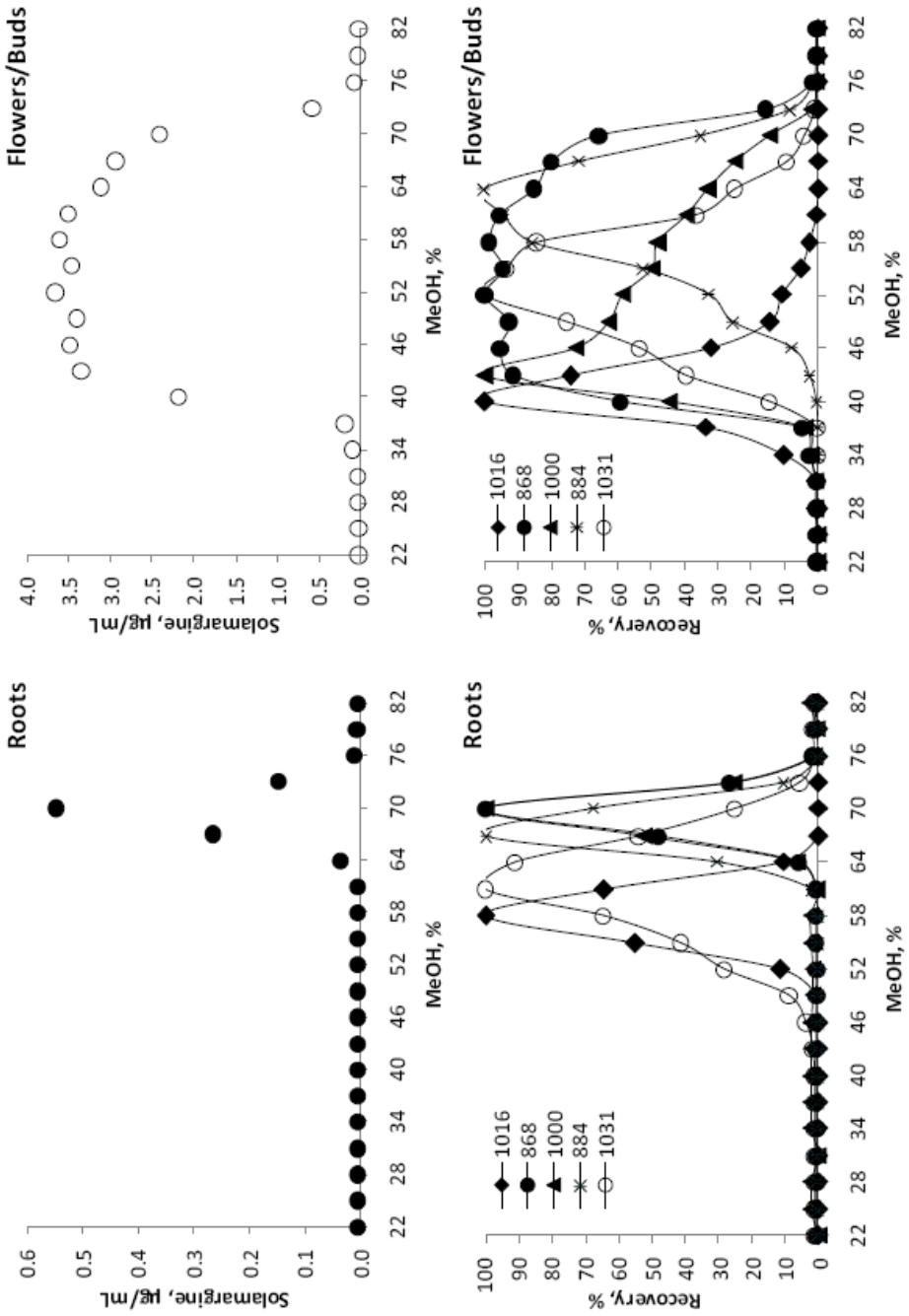

Figure 3.4. Elution of glycoalkaloids from methanolic plant extract on Waters® Oasis HLB solid-phase extraction cartridges with aqueous methanol at 3% step by collecting 3x2mL fractions.

Hydrolysis of Solanum sisymbriifolium extract

In general, hydrolysis of glycosides can be used as an aid to simplify chromatographic data, especially where appropriate standards are commercially unavailable and to minimize interferences in subsequent chromatography (Popova et al., 2009). In the case of glycoalkaloids, the use of hydrolysis is helpful for identification of aglycones. This, in turn, can aid in the identification of glycoalkaloids. Even if the molecular mass and molecular formula is the same for the aglycone, their structures can be different. The difference in structure would be reflected by the differences in aglycone retention times in the chromatogram of hydrolysate. On the other hand, if the aglycone for several glycoalkaloids is structurally the same, one peak with the peak area corresponding to the combined concentration will be observed.

Four detected glycoalkaloids (solamargine, sycophantine, hydroxysalamargine, and hydroxysolmargine) were hydrolyzed under acidic conditions to obtained free aglycones. Hydrolysis was done under reflux for 1, 2 and 4 hours (Fig. 22). Hydrolysis completion was monitored by the disappearance of glycoalkaloids and the formation of aglycones. While more than 90% of glycoalkaloid were hydrolyzed in just 1 h, the maximum yields of aglycones were only observed after 4 h, while partially hydrolyzed glycoalkaloids were predominant after 1 and 2 h (Fig. 23). The results are consistent with the previous studies where a series of glycoalkaloid standards were hydrolyzed under different conditions (van Gelder, 1984; Nikolic et al., 2005). It was demonstrated that it takes at least 2 h to hydrolyze solanine, solanidine, solasodine, demissine, and tomatine with 2 mM HCl (van Gelder, 1984). The hydrolysis appears to happen stepwise by consequent cleavage of sugar units (Fig. 22). For example, for solamargine, first one rhamnose unit is cleaved followed by the

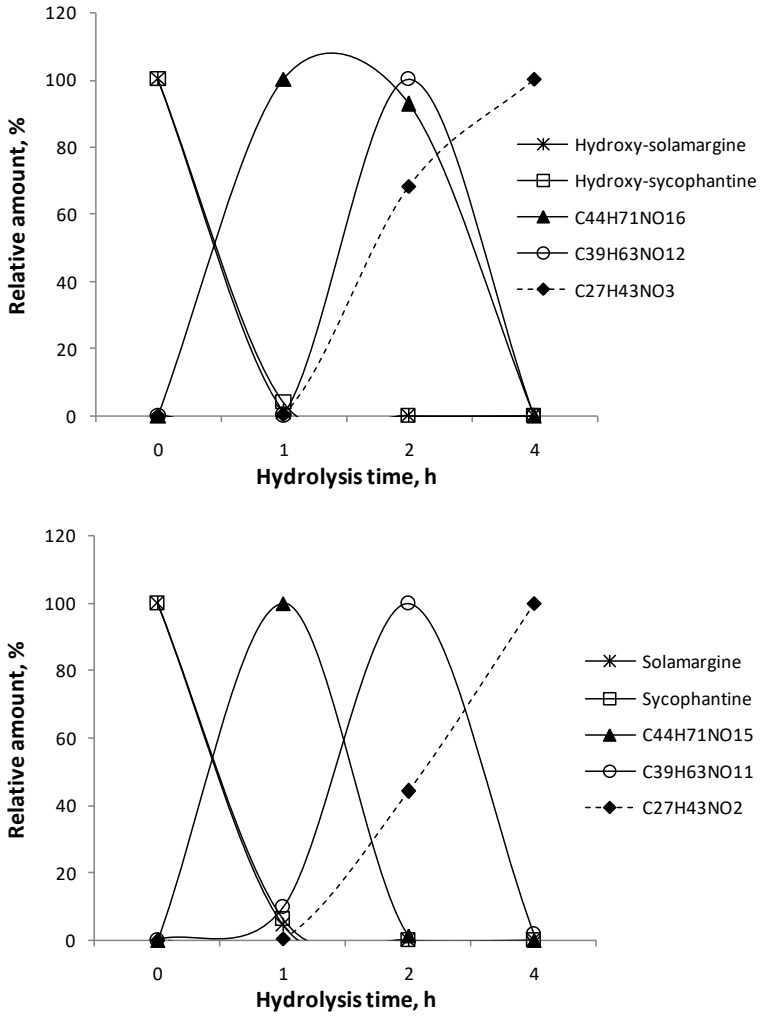

Figure 3.5. Time dependence of hydrolysis completion for four glycoalkaloids present in *Solanum sisymbriifolium* fruits/buds.

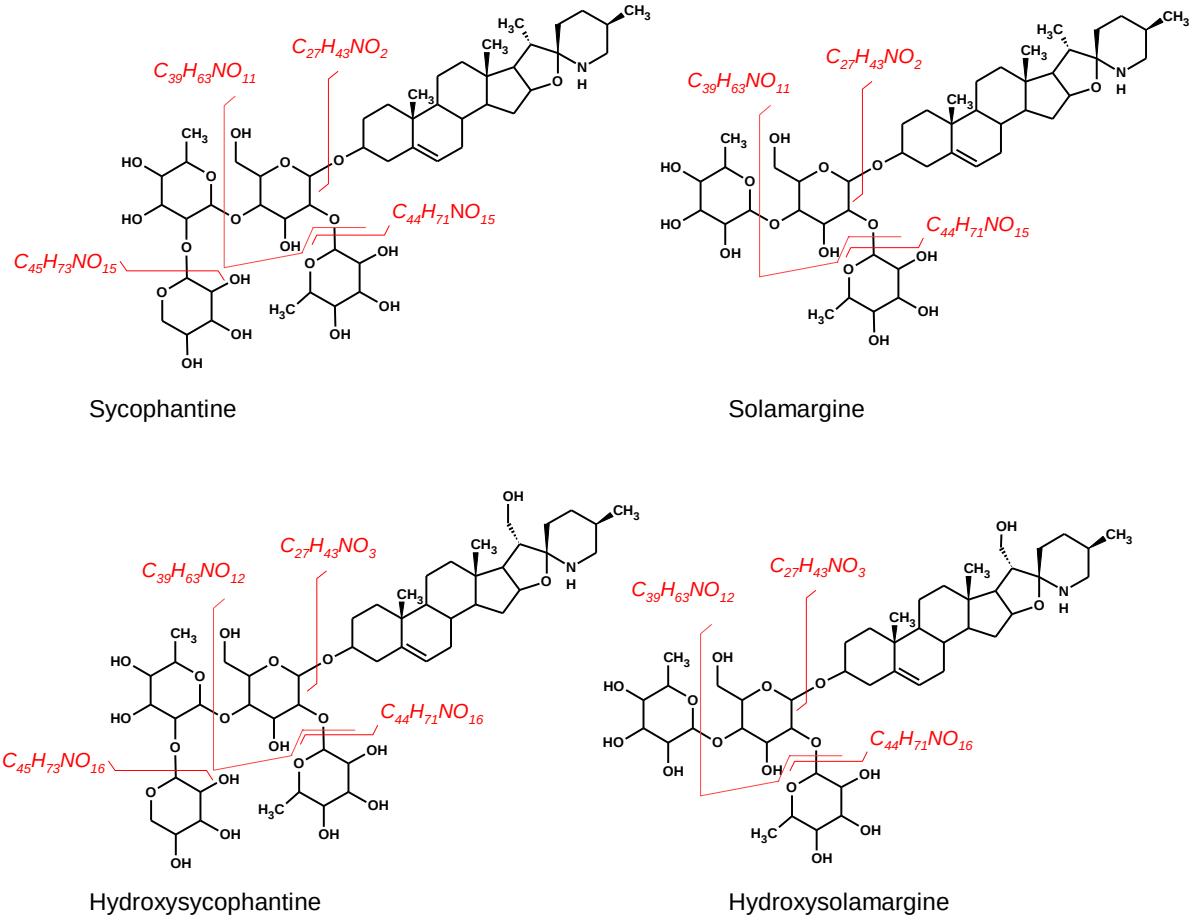

Figure 3.6. Schematic representation of acidic glycoalkaloids hydrolysis. Red line depict the bonds hydrolyzed with the corresponding molecular formulas of partially or fully hydrolyzed glycoalkaloids.

second unit. As a result, there is peak of solamargine-Rha ($C_{44}H_{71}NO_{15}$) molecule at 1 h of hydrolysis and peak of solamargine-rha-rha ($C_{39}H_{63}NO_{11}$) molecule at 2 h of hydrolysis. Similarly, hydrolysis of sychopantine proceed through the stepwise removal of sugar units. Hydrolysis of hydroxylated solamargine and sychophantine appears to differ in the removal preferences of sugar units. Specifically, at 1 h of hydrolysis glycoalkaloid with one rhanmose unit removed is dominant. At 2 h of hydrolysis, a mixture of glycoalkaloids with one and two rhamnose units removed is present unlike the case of solamargine, where glycoalkaloid with 2 rhamnose units removed dominates. For all glycoalkaloids after 4 h of hydrolysis only aglycones were present with no detectable concentration of intact and partially hydrolyzed glycoalkaloids.

When the chromatograms of fully hydrolyzed glycoalkaloids were analyzed, one peak with the molecular mass corresponding to each molecular formula of aglycone was found. Thus, it indicates that, indeed, solamargine and sycophantine have the same aglycone - solasodine. Hydroxysolamargine and hydroxysycophantine also produced only one peak with 430.33 m/z, thus suggesting that have common aglycone.

Metabolome analysis for specific plant organs

Obtained HPLC/MS chromatograms of flower, leaves, stems, and roots were analyzed by batch-processing feature extraction for differential analysis (Fig. 24). A total of 2886 individual compounds were identified with an almost even distribution among the plant organs: flowers (1622), leaves (1894), stem (1583), and roots (1285). Only 5% of detected compounds were shared among four organs, 26.2% were shared among at least three organs, 53.9% were shared among two organs, and 15 % were only present in one organ. Leaves

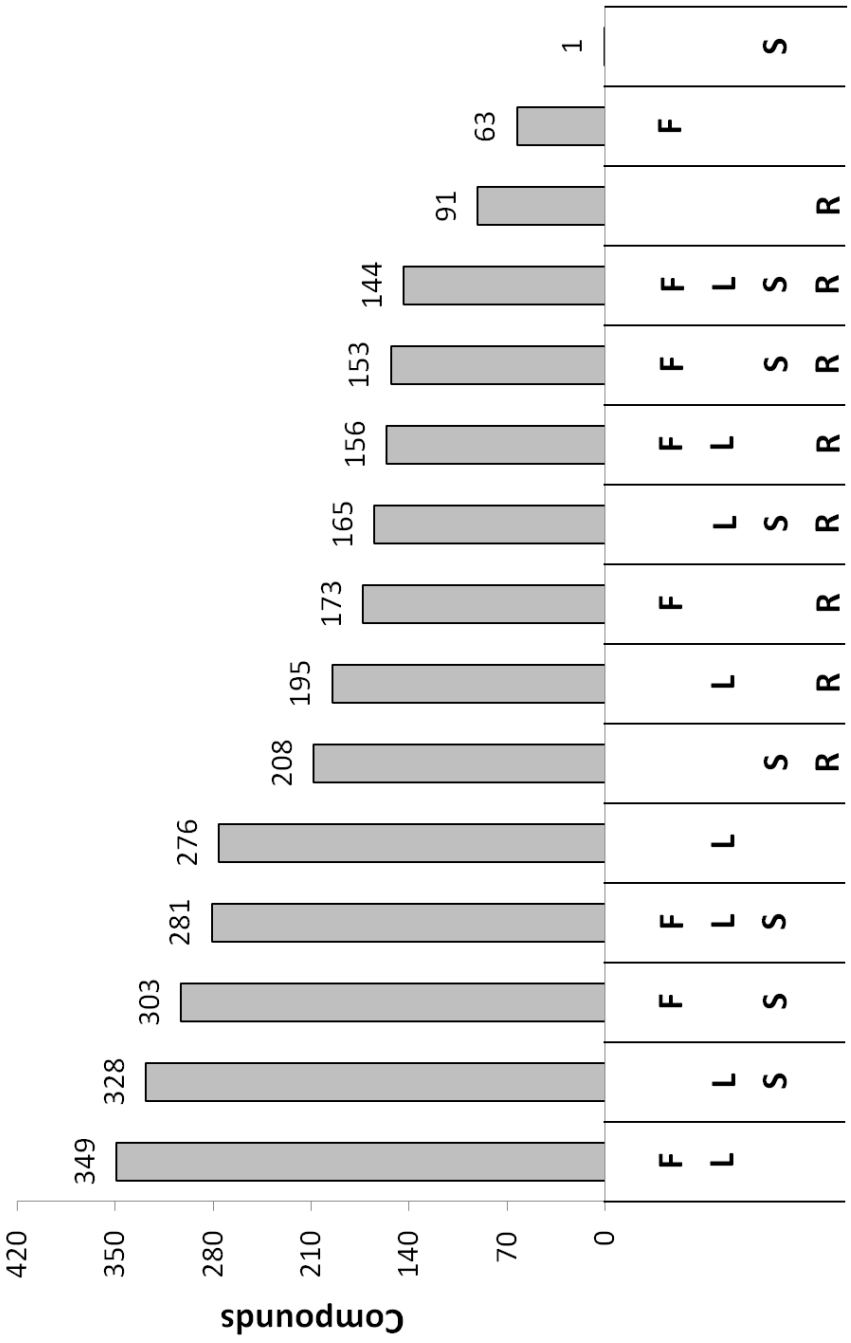

Figure 3.7. Individual compounds as identified by HPLC TOF MS in flowers (F), leaves (L), stems (S), and roots (R) in *Solanum sisymbriifolium*.

contained the largest number of unique compounds (276) followed by roots (91), flowers (63), and stem (1). When 60 most abundant peaks were visualized using heat map, the difference between chemical composition and the relative chemicals' amount of plant organs became clear (Fig. 25) (Metsalu and Vilo, 2015). For example, while leaves do have the largest amount of unique compounds, the relative abundance of these chemicals is lower as compared to stems and flowers. At the same time, stems have higher concentrations of at least 25% of all detected compounds. Similarly, flowers while have lower number of unique compounds, contained higher concentrations of chemicals (Fig. 25). Some of the detected compounds can be tentatively identified based on the molecular mass, expected chromatographic elution and literature references. However, only a limited number of metabolites was reported in *Solanum sisymbriifolium* making identification a challenge (Brown at al., 1999; Rodriguez-Perez et al., 2018). Nevertheless, a total of 19 glycoalkaloids were identified in plant extracts (Table 5). Of 19 glycoalkaloids, only two were present in all plant organs: solamargine and sycophantine. While roots had the least amount of glycoalkaloids, flowers, leaves, and stem had equal diversity of glycoalkaloids with different dominant glycoalkaloids. The difference in glycoalkaloids composition within the plant organs can be fundamental their biological activity. For example, it was demonstrated that *G. pallida* hatch is different when nematode cysts are exposed to leaves, flowers, or roots extracts of *S. sisymbriifolium* (Dundurand, personal communication, 2019).

Acetylcholinesterase assay

Acetylcholesterase assays were performed to evaluate the biological activity of *S. sisymbriifolium* fractions from flowers and roots. While it was demonstrated previously, that

Table 3.4. Distribution of tentatively identified glycoalkaloids in *Solanum sisymbriifolium* organs.

Glycoacaloid, m/z	Flowers	Leaves	Stems	Roots
765.489		+		
864.477				+
866.493	+			
868.510 (Solamargine)	+	+	+	+
882.487	+			
884.504 (Hydroxy-solamargine)	+	+	+	
954.509		+	+	
970.500		+		
1000.543 (Sycophantine)	+	+	+	+
1013.547	+			
1016.546 (Hydroxy-sycophantine)	+		+	
1031.546		+	+	+
1032.543	+	+	+	
1045.525			+	
1068.540				+
1069.522				+
1071.538	+	+	+	
1085.515	+		+	+
1117.546		+		

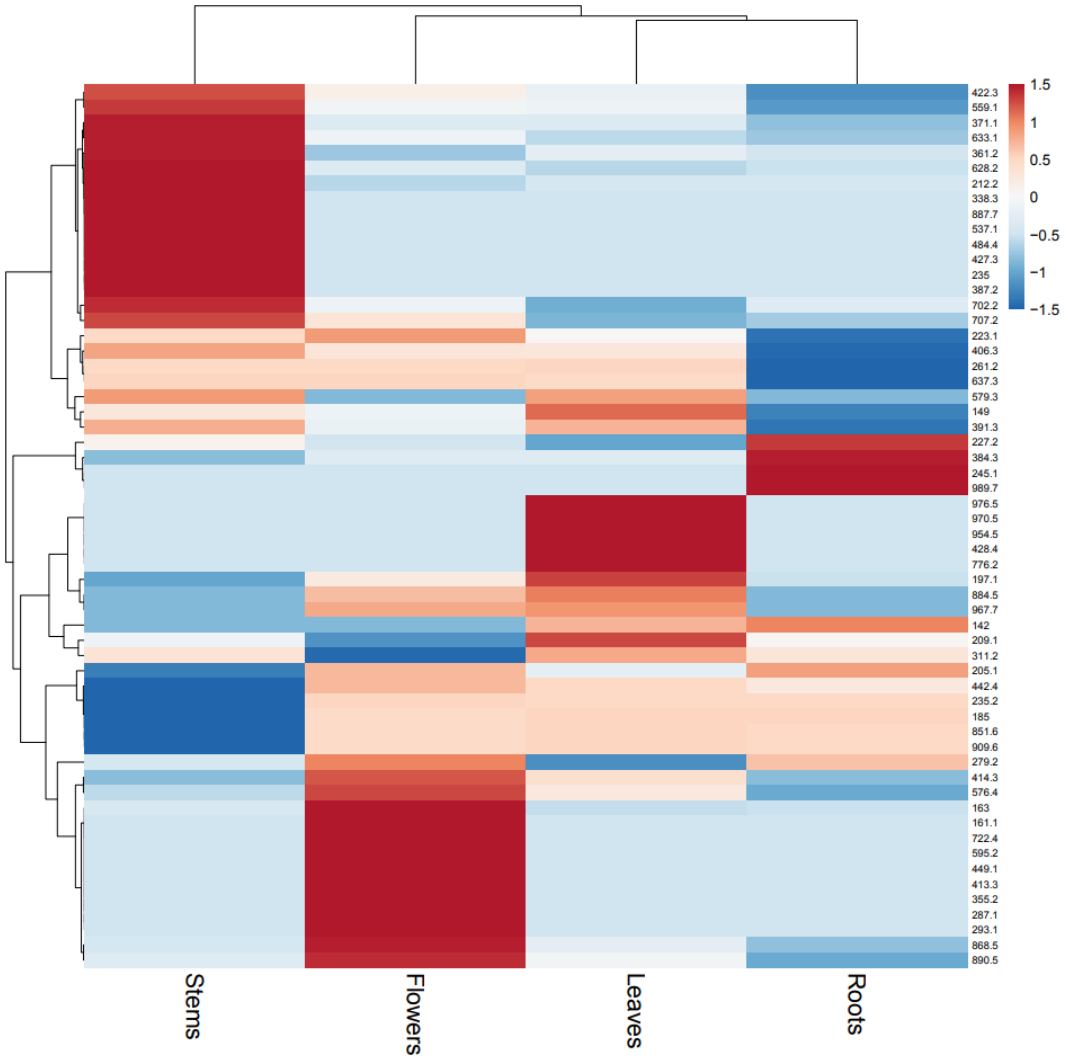

Figure 3.8. Heat map analysis of *Solanum sisymbriifolium* chemical composition for flowers, leaves, stems, and roots.

glycoalkaloids are acetylcholesterase inhibitors, no significant inhibition was observed in the present study (Fig. 26). When 23 methanolic fractions of roots or flowers/buds were tested as inhibitors, inhibition did not exceed approximately 60%. Even so, it appears that the inhibition effect was not specific as no dilution dependence could clearly be observed. At the same time, flowers/buds extracts affected the acetylcholesterase reaction rate to a greater extent. Specifically, 67 and 70% methanolic fraction of flowers/buds extract exhibited the highest inhibition effect among all tested fractions (Fig. 26). However, low inhibition of extract can be explained by the original low concentration of glycoalkaloids. For example, in the previous studies, concentration of glycoalkaloids were more than 100 times higher than glycoalkaloid concentrations in plant extracts (McGehee et al., 2000). Specifically, in the previous studies the concentration of glycoalkaloids that exhibit the inhibition of acetylcholinesterase was 10-100 µM (McGehee et al., 2000). However, the plant extract used in the present study contained only 0.02 - 5.5 µM of glycoalkaloids (Fig. 27). While the inhibition was observed with high glycoalkaloid concentration fractions, the overall expected effect from the plant extracts may be low. Thus, it would be hard to propose the acetylcholesterase inhibition is the major biological effect associated with *S. sisymbriifolium* extracts. At the same time, inhibition is a part of the biological response to *Solanum sisymbriifolium* extracts. However, acetylcholesterase inhibition assay cannotbe used for biologically guided fractionation of glycoalkaloids in *S. sisymbriifolium* extracts. Thus, alternative assays need to be developed.

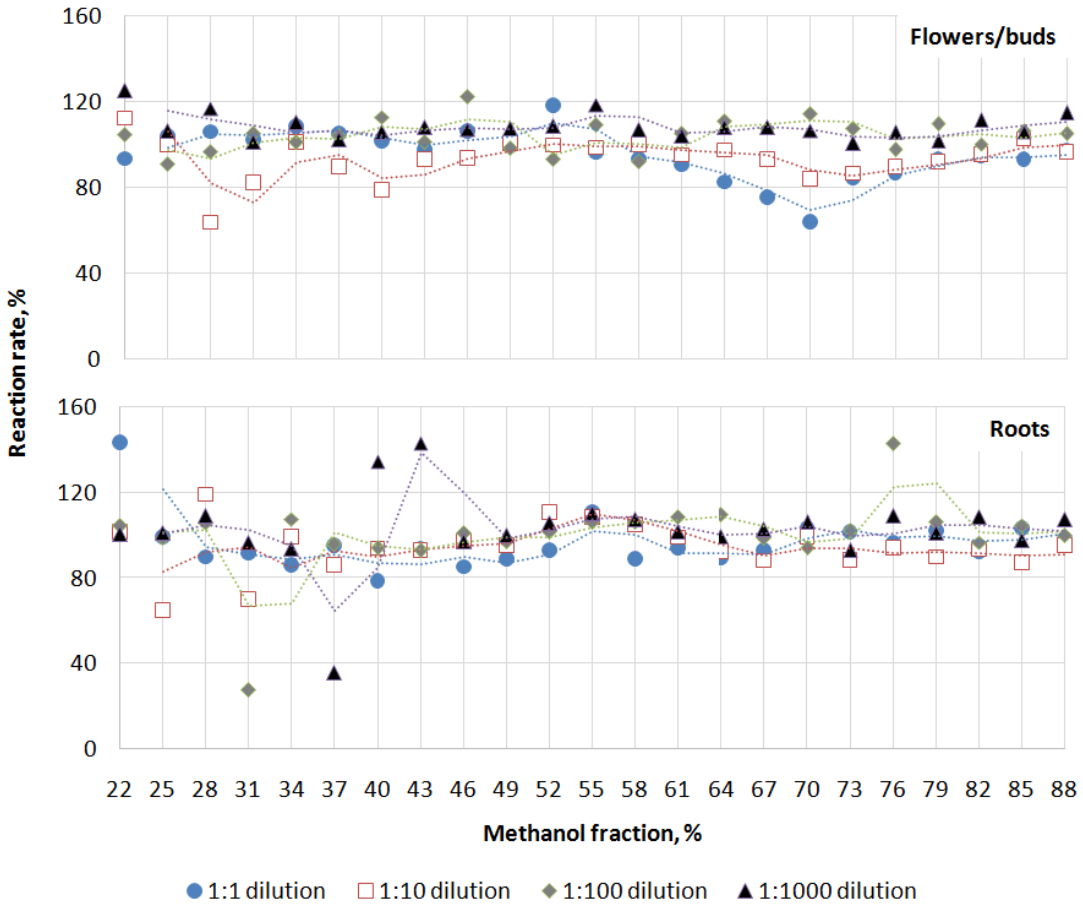

Figure 3.9. Reaction rate of acetylcholesterase catalyzed hydrolysis of acetylcholine in the presence of root or flowers/buds fraction obtained using different methanol percentage.

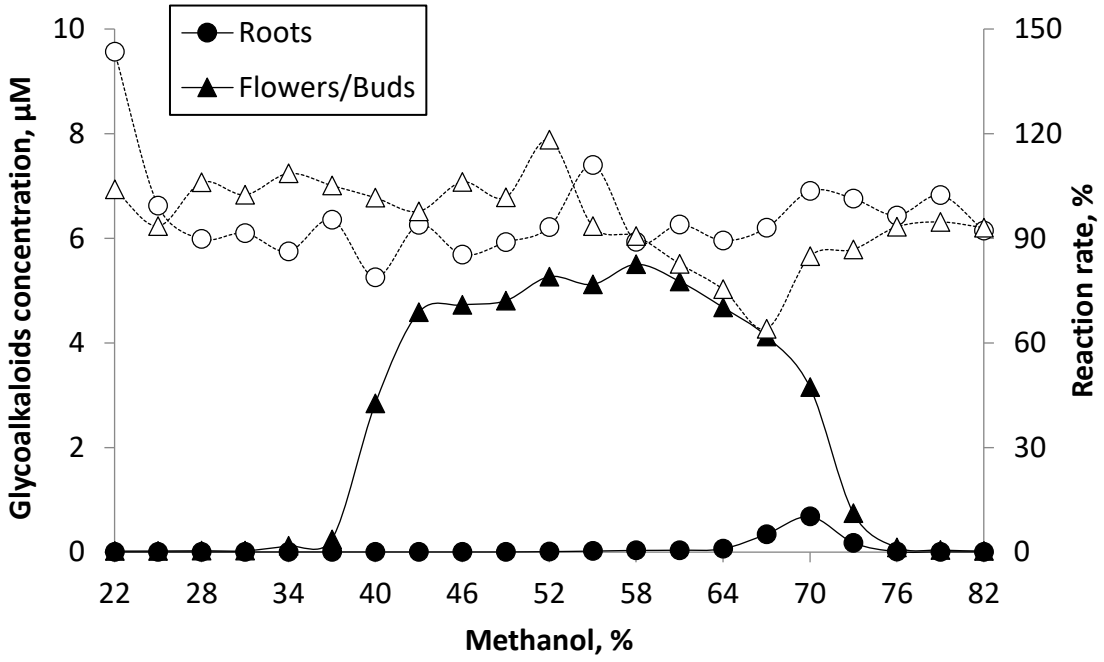

Figure 3.10. Reaction rate (open markers) of acetylcholesterase catalyzed hydrolysis of acetylcholine in the presence of root or flowers/buds fraction obtained using different methanol percentage. Cumulative concentration of glycoalkaloids are plotted with closed markers.

Concluding remarks

Based on the analysis of *S. sisymbriifolium* extracts, it is clear that research needs to be conducted further to gain understanding of the chemistry and potential pesticidal effects of glycoalkaloids of *S. sisymbriifolium*. The presence of a various glycoalkaloids within the plant and their relative ratio in different plant organs warrants further research. For example, it is still unclear what specific glycoalkaloids are present in the plant and which ones are responsible for pesticidal activity as well as possible synergy amongst the compounds. To address these questions, the chemistry of *S. sisymbriifolium* should first be elucidated. This can be doneby isolation of specific glycoalkaloids and analyzing them by chemical techniques such as NMR and IR. The structural analysis of glycoalkaloids may elucidate to the specific structural features that may be correlated with pesticidal activity, thus enabling better understanding of the mechanisms of pesticidal action.

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