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The Analysis of Ginsenosides in Ginseng Garden Soil

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Abstract

American ginseng (*Panax quinquefolius* L.) produces natural products called ginsenosides. The biggest challenge Ontario commercial ginseng farmers face is ginseng replant disease. To understand the function of ginseng root exudates, ginsenoside accumulation and persistence over time were investigated. Currently, no reliable ginsenoside specific extraction method that characterizes changes in soil chemistry exists. Ginsenoside extraction protocol optimization was required to determine how ginsenoside composition changed over time. Overall, protocol optimization resulted in a 30% increase in yield of ginsenosides compared to previous extraction protocols. In the ginseng gardens, ginsenoside accumulation occurred slowly and did not reach significantly measurable amounts until the end of the second growing season. Until that time, only trace amounts of ginsenosides were detected, but with no pattern of persistence. High levels of variation existed within sites, reflecting the non-uniform distribution of ginsenosides within garden soils. Future sample collection will solidify patterns seen in these fields.

Keywords

Ginsenoside, American ginseng (*Panax quinquefolius)*, extraction, soil, liquid chromatography mass spectrometry, ginseng replant disease, protocol development, soil chemistry, accumulation, persistence

Summary for Lay Audience

American ginseng (*Panax quinquefolius* L.) is grown for their health benefits. The health benefits come from compounds in the plants called ginsenosides. The commercial ginseng industry in Ontario produces a large income worth hundreds of millions of dollars. The biggest challenge Ontario commercial ginseng growers face is ginseng replant disease (GRD). Growers cannot successfully produce ginseng in the same garden after an initial crop. The disease is largely attributed to various harmful microorganisms; however, it is understood that GRD not only involves microorganisms. GRD also involves a combination of several abiotic and biotic factors that aren't well studied. It is becoming apparent that the compounds from ginseng plants, may play a role in this complex disease system. The objective of this project was to track ginsenoside accumulation and persistence in both newly planted and recently harvested ginseng gardens. To be able to determine changes in soil chemistry, such as the accumulation and persistence of ginsenosides, a reliable method of detection must be used. Currently, there is no consistent ginsenoside specific extraction method standard. Therefore, to accurately examine the soil for changes in ginsenoside composition, ginsenoside extraction protocol optimization and subsequent validation were required. Overall, improvements to the protocol were established, validated and applied to ginseng garden soils. Using the optimized protocol, ginsenoside concentrations were measured in newly planted gardens. The compounds accumulate to significant levels (relative to the control) after two years of growth. Trace amounts of ginsenosides were detected in the harvested gardens. There was no pattern of persistence in either of two sites monitored. Within site variation in ginsenoside content was evident. This was likely due to the non-uniform distribution of these compounds in the soil. Further collection and analysis of soils collected during the third and fourth growing seasons will allow for a more detailed analysis of pattern and trends described here. Ultimately, this project represents one piece of a puzzle that will add to our understanding of GRD. This research will show whether changes and composition in ginsenoside levels in newly planted and recently harvested ginseng gardens contribute to GRD.

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List of Abbreviations

ACN = Acetonitrile

ARD = Apple replant disease

CH_3CN = Acetonitrile

CITES = The Convention on International Trade in Endangered Species of Wild Fauna and Flora

COSEWIC = The Committee on the Status of Endangered Wildlife in Canada

DI = Deionized

ESI = Electrospray ionization

EtOH = Ethanol

FID = Flame ionization detector

GCMS = Gas chromatography mass spectrometry

GLM = General linear model

GRD = Ginseng replant disease

H_2O = Water

HCO_2H = Formic acid

HPLC = High performance liquid chromatography

HSD = Honestly significant difference

IMP = *Ilyonectria mors panacis*

LC = Liquid chromatography

LCMS = Liquid chromatography mass spectrometry

MeOH = Methanol

MS = Mass spectrometry

m/z = Mass to charge ratio

N_2 = Nitrogen

NaOAc = Sodium Acetate

NMR = Nuclear magnetic resonance

OGGA = Ontario Ginseng Grower's Association

PAH = Polycyclic aromatic hydrocarbons

PCB = Polychlorinated biphenyl

PPD = Protopanaxadiol

PPT = Protopanaxatriol

SARA = Species at Risk Act

SPE = Solid phase extraction

TOF = Time of flight

QTOF = Quadrupole time of flight

GLC = Gas liquid chromatography

TLC = Thin layer chromatography

1 Introduction

1.1 Ginseng natural products: Ginsenosides

Over time, plants have evolved numerous defensive mechanisms to combat a wide range of biotic and abiotic stressors in their environment. One such mechanism of plant defense is the development of secondary metabolites such as antimicrobial saponins (Papadopoulou et al., 1999; Mostafa et al., 2013). Saponins are innate defense compounds that are constitutively present in many plants and often act as protectants against biotic stressors such as pathogens (Papadopoulou et al., 1999; Yang et al., 2006). For example, it has been established that saponins in oats (avenacin A-1) are effective in deterring the disease caused by the fungal pathogen *Gaeumannomyces graminis* (Papadopoulou et al., 1999). The function of saponins as phytoprotectants has also been proposed for species in the genus *Panax* (Nicol et al., 2002), which produce saponins called ginsenosides. These compounds are unique to species in the genus *Panax* (Kim et al., 2015). Structurally, saponins consist of polycyclic aglycone core structure with one or multiple sugar side chains (Ma et al., 1999; Yuan et al., 2010). The aglycone is also referred to as a sapogenin and is either a steroid (C-27) or triterpene (C-30) (Majinda, 2012), with the latter forming the core structure of ginsenosides. The combination of the hydrophobic sapogenin and hydrophilic sugar side chains create the foaming “soap-like” characteristic of saponins (Majinda, 2012).

Ginsenosides are classified as dammarane, ocotillol, and oleanane types based on their core triterpene structure, as well as the type of sugar moieties present and sugar linkage positions (Yuan et al., 2010). Over 100 putative different ginsenosides have been identified, with dammarane triterpenes being most common (Cheng et al., 2007; Qi et al., 2011; Chen et al., 2019). Dammarane triterpenoids can be further divided into two groups: 20(*S*)-protopanaxadiols (PPD), such as Rb1, Rb2, Rb3, Rc, Rd, Gypenoside GXVII and F2, or 20(*S*)-protopanaxatriols (PPT), such as Rg1, Re and F11 (Wan et al., 2008). Ginsenosides are often subdivided into major and minor ginsenosides, indicative of their relative abundances in ginseng roots. For example, major ginsenosides found in American ginseng (Figure 1.1) include Re, F11, Rb1, Rd and Gypenoside GXVII, which

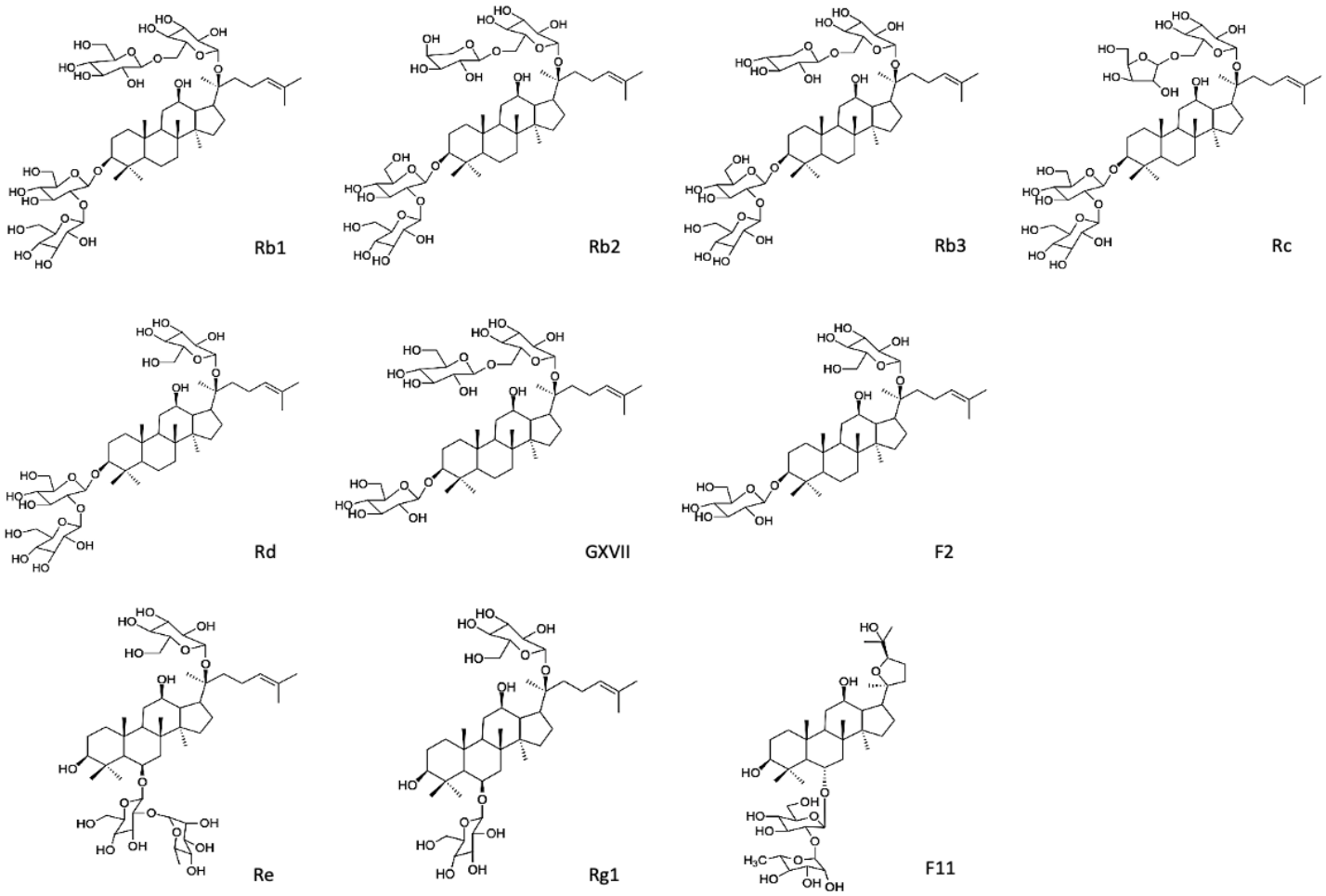

Figure 1.1. Common ginsenosides of American ginseng (*Panax quinquefolius*). 20 (S)-protopanaxadiols include Rb1, Rb2, Rb3, Rc, Rd, Gypenoside XVII, F2 and 20 (S)-protopanaxatriols include Re, Rg1 and F11

account for more than 70% of ginsenoside content in roots, whereas the minor ones include Rg1, Rc, Rb2, Rb3 and F2 (Court et al., 1996; Kim et al., 2005). Comparison of the types of ginsenosides found among different *Panax* species reveals that some *Panax* species have ginsenosides that are not found in others. One example of this is the presence of pseudoginsenoside F11 found only in American ginseng (*Panax quinquefolius*) and ginsenoside Rf distinct to Asian ginseng (*Panax ginseng*) (Schlag and McIntosh, 2006). Furthermore, the ratio of PPT and PPD differs between Asian and American ginseng, as does the total ginsenoside content (Schlag and McIntosh, 2006; Qi et al., 2011).

Both PPD and PPT are glycosylated steroidal triterpenes (Corbit et al., 2005). Most are bidesmosidic as they contain two saccharide side chains linked at different hydroxyl groups on the aglycone. In PPD, the side chains are located at the C-3 and C-20 positions, whereas in the PPT, the saccharides are located at the C-6 and C-20 positions (Schlag and McIntosh, 2006; Figure 1.2). Another feature differentiating PPDs and PPTs are the number of hydroxyl groups present. The PPD have 3 hydroxyl groups positioned at C-3, C-12, and C-20, and the PPT have 4 hydroxyl groups positioned at C-3, C-6, C-12, and C-20 (Yuan et al., 2010; Kim et al., 2015). The most common sugar moieties present include glucose, arabinose, xylose, and rhamnose (Shin et al., 2015). Differences in sugar decorations, stereoisomerism and attachment positions produce a diverse array of compounds.

As the technology and methodology for isolation of ginsenosides advances, more have been identified from *Panax* species. Ginsenosides have been isolated from various parts of the plant including the roots, fruits, leaves, stem and flower buds (Shin et al., 2015). Ginsenoside type and content varies across different parts of the plant, with larger amounts in the roots and leaves, followed by the stem (Yuan et al., 2010). For example, ginsenosides can account for ~3-7% and ~2-4% of the dry weight in roots and leaves, respectively (Court et al., 1996; Li et al., 1996). Ginsenoside content appears to increase with plant age (Court et al., 1996; Qu et al., 2009), making it preferential to cultivate the plant as long as possible before harvest (typically three to six years). In the soil,

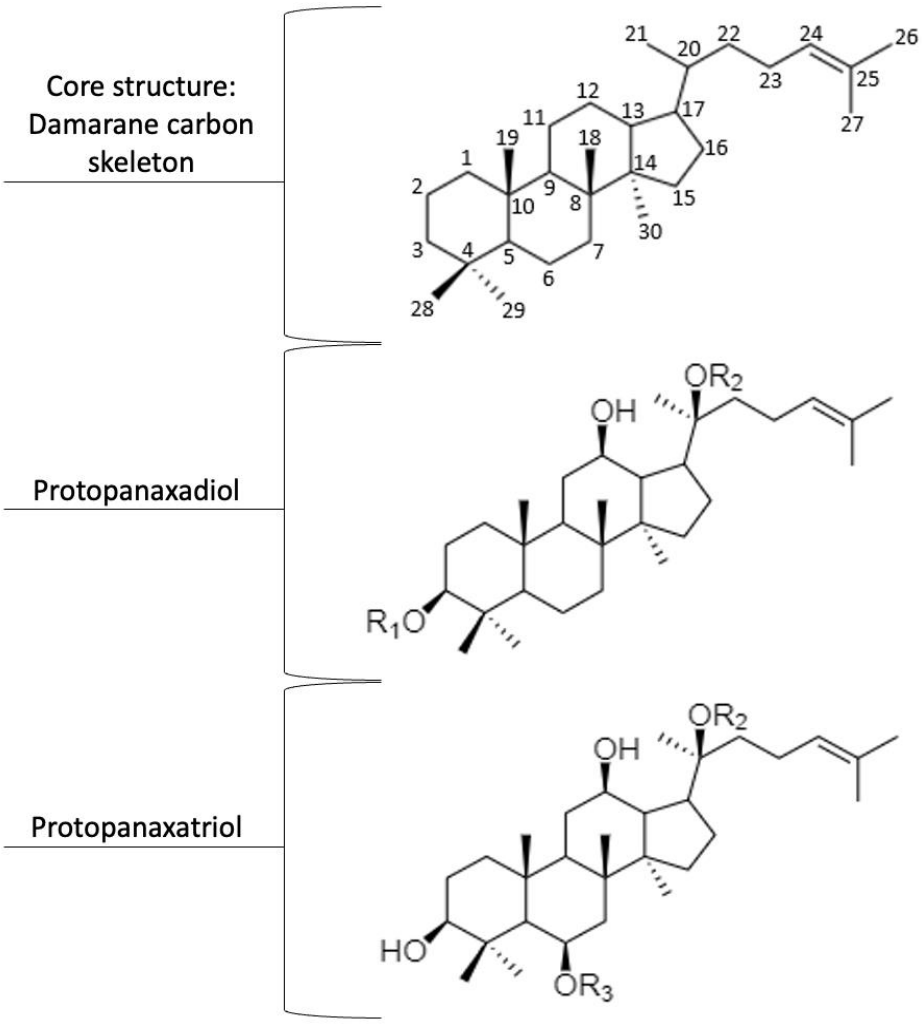

Figure 1.2. Carbon skeleton structures of ginsenosides found in American ginseng (*Panax quinquefolius*). The core structure is composed of 30 carbons and is divided up into two classes: protopanaxadiols (PPD) and protopanaxatriols (PPT).

ginsenoside profiles (content and total amounts) also vary, depending on factors such as plant age, cultivation practices, soil type and composition, climate and geographical location (Court et al., 1996; Lim et al., 2005).

Analysis of ginsenosides has involved various analytical methods including high performance liquid chromatography (HPLC) with either UV detection or electrospray ionization (ESI)-mass spectrometry (MS), including time of flight (TOF) (Ivanov et al., 2016) and quadrupole time of flight (QTOF) MS (Lee et al., 2017). Less common methods of measuring ginsenosides include gas liquid chromatography (GLC) and thin

layer chromatography (TLC) (Cui et al., 1993). These analytical techniques have helped in the determination of the structure of these compounds as well as their quantitation. Overall, the use of these techniques has improved our knowledge on the diversity of ginsenosides found in *Panax* species.

1.2 American ginseng: Morphology, history and cultivation

American ginseng, *Panax quinquefolius*, is a slow growing perennial herb that belongs to the Araliaceae family. Derived from the Greek word meaning "all healing", the genus *Panax* was first coined by Carl Anton von Meyer a Russian botanist (Leung and Wong, 2010). American ginseng thrives in wooded, shaded areas with loam-sand soil types, and is predominantly found in temperate climate zones (Charron and Gagnon, 1991; Yuan et al., 2010). In Canada, American ginseng is found in Ontario and Quebec, generally growing in the deciduous forests found in these regions (Charron and Gagnon, 1991). In the wild, ginseng plants can live up to 60 years (Charron and Gagnon, 1991). American ginseng is very scarce in its native range in North America, largely due to excessive harvesting. Its rarity combined with its significance as a medicinal plant has contributed to the disappearance of wild populations of American ginseng. Furthermore, habitat destruction of deciduous forests where sparse populations of ginseng are typically found, has further impacted these wild populations (Charron and Gagnon, 1991).

In 2000, natural populations of American ginseng were designated as endangered by the Committee for the Status of Endangered Wildlife in Canada (COSEWIC) and in 2003 American ginseng was listed with the same designation under the Species at Risk Act (SARA) (Carignan and Branchaud, 2018). This designation was established due to threats including illegal root harvest, habitat destruction (i.e. deforestation), disease and predation, forest harvesting, as well as climate change (Carignan and Branchaud, 2018). In 1973, American ginseng was included in the Convention of International Trade in Endangered Species (CITES), protecting American ginseng and regulating the import, export, handling and possession of the seeds and plants (Westerveld, 2010).

In Asia, ginseng has been cultivated for at least 2000 years (Lim et al., 2005). However, in Canada, commercial cultivation of *P. quinquefolius* has occurred over the past 100

years, following overharvesting of wild ginseng that led to population declines (Court et al., 1996; Westerveld, 2010). Commercially, ginseng is typically grown in high density shade gardens in primarily sandy loam, well-drained soil. In Ontario, planting of stratified seed occurs in the fall with emergence in the subsequent spring. Seeds are planted in raised beds and covered with straw mulch. The whole garden is then covered with overhead tarps that block ~70% of incident light to emulate shade conditions. The plants are cultivated for either three or four years; however, while the longer cultivation time is preferred, since the roots grow larger with age (Lim et al., 2005), progressive increases in disease year after year, often leads to collection at the end of the third year (Court et al., 1996).

Morphologically, a mature ginseng plant is characterized by a thick forked taproot (Charron and Gagnon, 1991) at the end of a rhizome from which annual stems emerge. The rhizome is decorated with scars that accumulate from annual abscission, as each year a new aerial stem is produced (Charron and Gagnon, 1991). The stem varies in height ranging from 5-60 cm depending on the age of the plant (Westerveld, 2010). Seedlings have one leaf, whereas mature plants have a whorl of leaves with each leaf consisting of a petiole and 3-5 compound, palmate leaves (Charron and Gagnon, 1991). As the plant matures, the number and size of the leaves increase. Flowering usually occurs when the plants are three years or older (Li, 1995; Westerveld, 2010). Once the plant flowers, each flower produces 1-3 seeds.

Market value for American ginseng is dependent on root shape and size. Roots are subdivided into categories based on their size and weight after drying (Li, 1995). Common grades used to categorize root shape include: spider, which have no distinct tap root, fiber which contain secondary or tertiary roots measuring 1-2 cm in length or less; forked, which have tap roots that range from 2-5 cm long with lateral root branching providing most of the dry weight; chunk, which also range from 2-5 cm in length with the tap root providing most of the dry weight; and finally pencil, which is a tap root that is greater than 5 cm in length (Roy et al., 2003). American ginseng is susceptible to root rot, which highly alters the shape and size of the root, and hence its quality. Root rot accounts for approximately 30-60% of yield loss of ginseng worldwide (Westerveld, 2010).

The ginsenosides within ginseng plants, particularly in *Panax ginseng* (Korean ginseng), *Panax notoginseng* (Chinese/Sanqi ginseng), and *Panax quinquefolius* (American ginseng) are highly sought after and subsequently cultivated for their pharmacological and medicinal properties (Li, 1995). Numerous studies have reported the medicinal benefits of ginseng and the positive effects it has on the cardiovascular system (Ding et al., 1995), immune system (Predy et al., 2006), central nervous systems (Yuan et al., 2010; Qi et al., 2011) as well as anti-diabetic effects (Oh et al., 2014). For this reason, the demand for ginseng has increased, especially in North America. In Ontario, the Ontario Ginseng Growers Association (OGGA) report that the commercial ginseng industry had an annual farm gate value of approx. $250,000,000 between 2014-2017, with 95% exported to Asia. The high value of this specialty crop and its economic importance to the agriculture industry in Ontario drives continued production. However, the future of American ginseng commercial production in Ontario remains uncertain.

1.3 Replant disease

Replant disease, also known as soil sickness, in agriculture is a detrimental issue often causing major economic losses to growers. A common theme amongst crops that experience replant disease is low yields, high mortality rates and/or reduced productivity. Replant disease is often tightly associated with soil health, which is influenced by a range of biotic factors such as soil microorganism composition, and abiotic factors such as soil fertility, organic content, and allelochemical deposition from existing or residual crop debris. The biggest challenge commercial ginseng growers face in Ontario is ginseng replant disease (GRD). Losses to GRD result from a decline in germination, poor growth and severe disease in seedlings planted to soils in which a previous ginseng crop had been cultivated (Yang et al., 2015; Farh et al., 2018). This in turn produces reduced marketable yields and/or crop failure. One of the confounding problems of GRD is its persistence, since GRD conditions are known to persist for decades. Consequently, growers cannot successfully cultivate ginseng in the same garden more than once, leading to a decline in the amount of available arable land suitable for ginseng cultivation. Well-drained sandy fields necessary for optimal ginseng production, but not previously used to grow ginseng, are becoming scarce in Ontario. The OGGA has estimated that Ontario

will run out of suitable land for commercial ginseng production in the next 20-30 years, or even earlier.

Ginseng seeds are sown in the fall, and require three to four years of growth before roots are harvested. This extensive growing period results in the development of GRD conditions which manifest in subsequent plantings. The main biological contributor to replant disease in American ginseng is *Ilyonectria mors panacis* (IMP). The buildup of inoculum from IMP and other organisms such as *Pythium irregulare* (Rahman and Punja, 2005), in conjunction with the buildup of allelochemicals produced from ginseng, are suspected to lead to overall decline in soil health during the initial cultivation of ginseng in a garden. Together, these factors contribute to a rampant replant disease that affects the seed and seedling survivability in subsequent plantings.

Generally, replant disease has been mainly attributed to biotic factors such as pathogens (Mazzola and Manici, 2012). Pathogens that affect ginseng, such as *Pythium* spp and *Ilyonectria* spp are also known to be pathogenic against apples and contribute to apple replant disease (ARD) (Braun, 1995). However, there is evidence that replant diseases may involve a complex of both abiotic and biotic factors. Like ginseng replant disease (GRD), apple orchards experience apple replant disease (ARD). In ARD, the replanting of young apple trees at the same site as previous apple trees can result in disease in the new trees with symptoms such as stunted trees, reduced fruit yields, root damage and inhibited shoot and root growth (Braun, 1995; Winkelmann et al., 2019). Simon et al., (2020) demonstrate that pathogen survival is related to abiotic soil properties and that these abiotic soil properties can lead to greater severity of ARD. Winkelmann et al., (2019) determined that soils that are predominantly sandy in composition are more susceptible to ARD. Notably, the same soil type is typical of ginseng gardens. Furthermore, the apparent combined influence of biotic and abiotic factors that are thought to contribute to ARD may account for the severity of apple replant disease. These factors could be mirrored in GRD and help explain the longevity and severity of this disease as well. Further understanding the role that abiotic factors like allelochemicals and soil properties play in replant disease and their interaction with biotic factors can strengthen our knowledge on this phenomenon and inform ways to mitigate it.

1.4 Plant pathogen interactions

American ginseng is exposed to a variety of fungal pathogens in the ginseng gardens, including foliar pathogens such as *Alternaria panax, A. alternata, Botrytis cinerea*, while other pathogens target the root and seed such as *Rhizoctonia solani, Pythium irregulare, Cylindrocarpon destructans* (re-classified as *Ilyonectria mors-panacis*) and *Fusarium spp.* (Reeleder and Brammall, 1994; Punja, 1997; Reeleder et al., 2002). Nicol et al., (2002) established that ginsenosides from American ginseng (*P. quinquefolius* L.*)* are mildly fungitoxic *in vitro*, indicating their potential as defense compounds and phytoprotectants against fungi such as *A. panax*. However, the relationship between these compounds and fungi is not straight forward. As the expansion of ginseng production occurred in Canada, so has the prevalence of these foliar, seedling and root diseases (Punja, 1997). The most destructive pathogen and suspected leading biological agent in GRD is the soil-borne pathogen *Cylindrocarpon destructans*, now known as *Ilyonectria mors-panacis* (IMP) (Farh et al., 2017, 2018). *Cylindrocarpon destructans*, was initially reclassified into 4 different genera: *Neonectria/Cylindrocarpon, Rugonectria, Thelonectria* and *Ilyonectria* (Farh et al., 2017). Of these genera, IMP was identified as the most aggressive isolate causing the most severe root rot disease in ginseng (Farh et al., 2017). However, it is understood that GRD involves not only IMP but a combination of abiotic and biotic factors. It is speculated that the exudates from ginseng plants, specifically ginsenosides and breakdown products from associated residues, may play a role in the establishment of this complex disease system (Bernards et al., 2010).

It is known that ginsenosides have mild fungitoxic effects against certain fungi, but are stimulatory to some ginseng pathogens (Nicol et al., 2002, 2003), which may create a double-sided effect of these compounds as contributors to GRD. As the remaining root and plant debris left over from harvest decomposes over time, more ginsenosides and/or their breakdown products are released into the soil. As these compounds accumulate and break down in the soil, they may differentially affect the soil microbes, favouring the growth of IMP and other pathogens. Li et al., (2020) describe this, as they looked at the influence between autotoxic ginsenosides and their effect on soil fungal microbiome diversity. They found that addition of autotoxic ginsenosides to soil that Sanqi (Chinese)

ginseng was grown in, altered the composition of the fungal microbiome. More specifically, they found that fungi pathogenic to Sanqi ginseng, such as members of *Fusarium, Cylindrocarpon,* and *Alternaria*, increased in abundance in soils with ginsenosides and taxa known to be beneficial, such as from the genera *Ochroconis, Acremonium,* and *Mucor,* decreased in abundance. Li et al., (2020) highlight the ability of autotoxic ginsenosides to influence and cause changes in microbial communities that are typical of ginseng soils. The potential presence of ginsenosides and/or crop residue could create an environment where pathogens are drawn to the breakdown products. This can alter the composition and growth of these organisms, and subsequent replanted crops and newly planted seedlings become more susceptible to this disease (Broeckling et al., 2008; Yang et al. 2015). Ginseng roots are typically harvested after 3-5 years of cultivation, corresponding to 3-5 years in age, and it is during this period the plant is susceptible to disease (Seifret et al., 2003).

Ginsenosides may act as chemoattractants for IMP as well as other microorganisms (Punja, 1997; Nicol et al., 2003; Zhang et al., 2011; Ivanov et al., 2016). Microorganisms such as *Pythium irregulare* have the ability to biotransform ginsenosides through deglycosylation (Yousef and Bernards, 2006), making them more biologically available and/or active, consequently reinforcing the potential involvement of ginsenosides in GRD (Ivanov and Bernards, 2012). Additionally, specific ginsenosides have exhibited autotoxic effects to ginseng, contributing to the susceptibility of young plants to pathogen attack (Zhang et al., 2011; Yang et al., 2015). Furthermore, *in vitro*, ginsenosides have been shown to induce and stimulate the growth of IMP (Nicol et al., 2002). Understanding GRD and the contributing abiotic and biotic factors that make it so severe are critical to ensuring the survival of the ginseng industry in Ontario. There are many suspected factors at play that underlie GRD, including ginsenosides.

1.5 Allelopathy and autotoxicity

There have been numerous recorded instances of plant species from a wide range of taxa exhibiting allelopathic potential on other plant species. In 1984, botanist Elroy Rice solidified the concept of allelopathy as "*any direct or indirect (positive or negative) effect by one plant on another plant, through the production of chemical compounds that are*

released into the environment" (Rice, 1984). Since then, the definition of allelopathy has diverged to become more specialized and specific. As it stands, there are several subtypes of allelopathy (Dahiya et al., 2017), primarily, interspecific and intraspecific allelopathy. Interspecific allelopathy involves allelochemicals produced by one plant (i.e. the donor plant), that are toxic to a different species (i.e. the target plant). Conversely, intraspecific allelopathy refers to the allelochemicals produced by a donor plant that are toxic to itself and to its own species. When a plant produces compounds that are toxic to itself, this phenomenon is called autotoxicity. Both interspecific and intraspecific allelopathy have been documented in agroecosystems and in the natural environment, such that Singh et al., (1999) report that both weeds and agricultural crops are known to display these phenomena. Both American ginseng and Chinese ginseng are crops that exhibit intraspecific allelopathic effects as they produce autotoxic compounds (He et al., 2009; Yang et al., 2015).

Dahiya et al., (2017) describe other forms of allelopathy, such as true and functional allelopathy. These two types refer to the mode of release and subsequent method of toxicity (i.e. how the compounds become toxic). Functional allelopathy occurs when once a plant produces allelochemicals and they enter the environment, the compounds become toxic due to modifications by microorganisms. Alternatively, in plants that display true allelopathy, the allelochemicals produced by the plant are naturally toxic.

Allelochemicals are known to have spatial effects. These effects can be categorized into two types: direct and residual allelopathy (Dahiya et al., 2017). Residual allelopathy is described as the left over and break down of plant debris that accumulates and succeeding plants that grow on the same area of land become affected due to the presence and release of allelochemicals from left over plant debris. However, in direct allelopathy, allelochemicals released from the donor plant directly affect the target plant(s) that are within the vicinity of the donor plant.

Plants produce and synthesize a diverse range of primary and secondary metabolites. Many types of secondary metabolites are known to display autotoxic effects, including some terpenoids and steroids (Li et al., 2012), glycosides (Yang et al., 2015), phenols

(Chou and Lin, 1976, He et al., 2009), coumarins (Kato-Noguchi et al., 2017), and flavonoids (Dornbos et al., 1990). The deposition of secondary metabolites within a plant and their respective concentrations can vary among species of plants. Kruse et al., (2000) report allelochemical accumulation in rhizomes, roots, seeds, stems, leaves and flowers. The concentration and deposition of these compounds in plant tissues can impact how these compounds enter the environment. For example, allelochemicals can be released into the environment through root exudation, volatilization, leaching via abiotic or biotic mechanisms, and through decay and decomposition of plant residues (Albuquerque et al., 2011). Various factors such as the developmental stage of the plant, plant organ, concentration of the released compound, climate and season determine the degree of toxicity of the allelochemical produced and released into the environment (Einhellig, 1996). In the case of ginseng, the highest proportion of ginsenoside content is found in the roots. Ginsenosides may enter the environment through root exudation (Nicol et al., 2003), but could also enter the soil through the decay and decomposition of plant debris left over from harvest. Root exudates coupled with decay of plant debris left over from harvest can contribute to ginseng field soils containing trace amounts of these compounds. Interestingly, Ben-Hammouda et al., (2001) found that for barley (*Hordeum vulgare*) extracts, the growth stage and plant organ influenced the degree of inhibitory effects of the extracts on bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*). It is evident that the presence of these compounds in soil have an array of effects within their respective agroecosystems.

The production of secondary metabolites, including allelochemicals, may occur in response to various stressors, in addition to the baseline amounts innately present in these plants. In an agriculture setting or in the natural environment, plants are subject to a variety of biotic stressors like pathogens, competition, and herbivory, as well as abiotic stressors like flooding, drought, and fluctuating temperatures. The production of allelochemicals and their effect on a target plant are influenced by these abiotic and biotic factors (Einhellig, 1996). A single mode of stress or combination of stressors on a plant, can alter the production of allelochemicals, leading to increased exudation into the environment. To highlight this, wild ginseng typically grows in sparse populations, with large distances between plants. It is possible that mature plants exude ginsenosides to

inhibit the growth of other ginseng seedlings within the vicinity of the mature plant; reducing competition. The activity that occurs between an allelochemical and its effect on a target plant is a dynamic interaction. This interaction not only involves both the physiological and ecological properties of the donor and target plants, but more importantly the interaction between the compound and the soil in which the plants are rooted in (Kobayashi, 2004).

In agricultural settings, autotoxicity and allelopathy are commonly observed (Weston and Duke, 2003). While interspecific allelopathy may be beneficial in an agricultural setting, other types, mainly intraspecific allelopathy or autotoxicity, are often costly and detrimental to a crop. A key player in the severity of allelopathic interactions is soil. It is the interface that bridges the donor plant to the target plant. Allelochemicals usually end up in the soil and can contribute to changes in the soil chemistry and environment. As allelochemicals enter the soil, factors such as soil type, moisture content, microbial presence (fungi, oomycetes, bacteria etc.) can impact the effectiveness, severity and availability of the allelochemical(s) on the target plant (Kruse et al., 2000).

The accumulation of detrimental allelochemicals in agriculture soil is a term called soil sickness, also referred to as soil fatigue (Singh et al., 1999). These compounds can alter the soil chemistry affecting the general health of the soil and its ability to support the growth of plant species. One proposed role of autotoxicity in nature has been thought to have evolved as a method to maintain spatial balance in plant communities. For example, mature plants may be better able to produce autotoxic compounds and therefore outcompete younger plants of the same kind for resources such as water, light and nutrients (de Albuquerque et al., 2011). However, in agroecosystems, soil sickness may be exacerbated by crop residues and root debris being left over post-harvest from the high density of these crops being grown in a given area. The accumulation of plant debris and/or the allelopathic compounds in the soil result in declines in yield after continuous harvest year after year, furthering the damaging effects of autotoxicity and soil sickness within a field (Singh et at., 1999). Yu and Matsui., (1997) and Yang et al., (2015), describe that the release of allelopathic compounds can affect the success of the next crops planted in a field (to varying degrees), and create imbalances in nutrient

availability, microbial communities and soil chemistry. Aside from American ginseng, there are numerous other crops that display autotoxicity including: Chinese/Sanqi ginseng (*Panax notoginseng*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), alfalfa (*Medicago sativa*), and asparagus (*Asparagus officinalis*) (Singh et al., 1999).

The ginsenosides produced by ginseng are known to display autotoxicity as they hinder the emergence and growth of Chinese ginseng seedlings (Yang et al., 2015). Remedies for this problem are difficult as successful cultivation requires 30 plus years of crop rotation (Yang et al., 2015). He et al., (2009) found similar effects of autotoxicity for phenolic compounds extracted from the fibrous roots of American ginseng, as they reduced the growth of seedlings in a concentration dependent manner. He et al., (2009) also verified the presence of these autotoxic phenolics in the field soils where American ginseng was cultivated, further supporting the evidence of the impact these compounds have on crop yield. When rice fields are left fallow, rice straw often gets left behind and decomposes in the fields. Chou and Lin, (1976), isolated allelochemicals from these rice residues, namely phenolic compounds such as ferulic acid (FA), *p*-coumaric acid and *o*-hydroxy phenyl acetic acid, from the soil. It is known that these compounds, particularly FA, can inhibit root elongation in rice seedlings and formation of root hairs, lateral roots and crown roots (Chi et al., 2013). Like ginseng, alfalfa experiences replant disease as these plants contain water soluble (phenolic) compounds that are both autotoxic and allelopathic. *In vitro*, Chon et al., (2002) demonstrated that alfalfa leaf extracts containing coumarin, *o*-coumarin, hydroxy-cinnamic acid and trans-cinnamic acid reduced root lengths of alfalfa seedlings and caused stunted and swollen root tips of these seedlings. Another common example of this phenomenon involves asparagus. It is known that asparagus root residues that remain in soil during asparagus cultivation can inhibit the growth of this plant. Kato-Noguchi et al., (2017) report that asparagus rhizomes contain allelochemicals that are toxic, such as *p*-coumaric acid, and *iso*-agatharesinol. By application of aqueous asparagus rhizome extracts on asparagus seedlings, the growth of these seedlings was inhibited, and application of these compounds inhibited the root and shoot growth of asparagus. These are just a few examples of plant species that exhibit allelopathic and autotoxic effects, impacting the succession of crops replanted in their

respective fields. What sets ginseng apart from these other species is the longevity of the rotation that needs to occur to produce successful yields.

1.6 Natural product extraction and protocol optimization

Plant diseases continue to be a limiting factor in agriculture. Plant health is greatly tied to and affected by processes that occur in the rhizosphere. Therefore, it is important to understand both the abiotic and biotic properties of soil that contribute to the decline of plant health. Agricultural soils are heterogeneous in nature and vary in chemical and physical properties. Crop rotation and farming practices can greatly modify properties of soil such as the size, shape and state of aggregates in cultivated soil, which can impact the relationship between soil, water and air (Carter, 2004). The variabilities in soil properties like organic matter and nutrients, clay content, and soil moisture content, allow for the growth of crops but also influences the incidence of soil diseases like soil sickness (Simon et al., 2020).

From a research standpoint, the complexities of the soil matrix can create difficulties in extracting compounds. Capriel et al., (1986), report that bound pesticide residues in soil are not typically detected during residue analysis. This raises the point of developing rigorous techniques in extracting compounds that are tightly bound to soil. These issues are long standing, and still require work to improve and reduce protocol inefficiencies. The soil matrix is quite heterogeneous, with numerous and varied binding sites between particle pores, and microregions that create opportunity for organic compounds to bind to and remain in soil. For example, Northcott and Jones, (2000a) highlight the difficulty in extracting polycyclic aromatic hydrocarbons (PAH) from the soil matrix. Their aim was to re-evaluate numerous spiking procedures and establish an optimal spiking procedure. The reasoning for undergoing these trials was influenced by established knowledge that PAHs are subject to losses during sample work up (reaction and processes), but also that levels stay bound to soil and sediment. Northcott and Jones, (2000a) undertook rigorous testing to determine the optimal conditions for spiking soil with PAH, including comparison between wet and dry soil, different solvents and respective volumes and various methods to produce thorough distribution and homogeneity of the soil mixture. Their research highlighted that with thorough testing and subsequent validation, high

recovery of organic compounds, in this case PAH, was achievable, despite the complexity of the soil matrix. The issues described above apply equally to the extraction of ginsenosides from ginseng garden soil in an agriculture setting.

Research that involves the determination and quantification of organic compounds in environmental media, such as soil, typically involves some emulation of natural conditions but in a lab setting. This is the case when chemical compounds are being evaluated for factors such as their persistence, bioavailability, toxicity and biodegradability in environmental media (Northcott and Jones, 2000b). The process of evaluating the above parameters involves a spiking trial, defined as the addition of a test material (such as a chemical or mixture of chemicals) to a clean, control/reference material, and subsequent mixing and homogenizing the two materials (Northcott and Jones, 2000b). The spiked soil can then be evaluated for compound toxicity, availability, and persistence (Northcott and Jones, 2000b) under conditions in which the target compound(s) concentration is known. By extension, the efficacy of soil extraction for the purpose of quantifying target compounds can be evaluated when the amount of the target compound in the environmental sample is known. The process of optimizing a protocol to quantify the presence and persistence of ginsenosides in ginseng garden soil, spiking trials can be used to evaluate extraction efficiency.

In most cases, natural products are compounds with molecular weights < 2000 amu (Sarker et al., 2006). It is commonly known that there are drawbacks in precisely extracting natural products, especially from complex matrices such as soil. Some of the drawbacks include low/reduced selectivity, low extraction yields, labour intensity, and problems with automation and efficiency, which lead to low reproducibility. Regarding problems with extracting saponins like ginsenosides, many of these compounds have similar structures, only differing in the side chains present and polarities. Due to this, it becomes challenging to distinguish them chromatographically (Majinda, 2012). Overcoming these challenges requires rigorous optimization trials, tests and developments. With time invested in optimization, the improved extraction technique can lead to greater yields, higher efficiency and increased reproducibility, resulting in savings of time and money, as well as reliable data.

When choosing an extraction procedure for natural products like ginsenosides, various aspects need to be considered. One must consider the target of the extraction (i.e. if the compound is known or unknown, if an array will be isolated or a singular group of interest etc.). The goal of the extraction should be identified as well, such as whether the purpose is to purify a certain amount, partially or fully and at what level of purity. Once these are answered, the following considerations are typical of natural product extractions. First, homogenization, drying and/or grinding, as well as the physical nature of the material must be considered. Following this, the solvent for extraction should be chosen, taking into account the polarity of the target compounds and the wettability of the material/matrix to be extracted. Relatively polar solvents such as ethanol, methanol and water, are frequently used (Rostagno and Prado, 2013), though compound solubility, cost, and selectivity should be considered when choosing a solvent (Zhang et al., 2018). A gradient of solvents may be used for increased extraction efficiency (Sarker et al., 2006). The greater the ratio of solvent to material, the higher the yield, but using excess solvent will result in a long period of time to concentrate extracts (Zhang et al., 2018), reducing efficiency.

Next, an extraction method is tested, with some options including maceration (i.e. using a gyratory shaker), boiling, supercritical fluid extraction, soxhlet, and distillation to name a few (Sarker et al., 2006; Zhang et al., 2018). The extraction method produces a crude extract. While in some cases the crude extract can be taken straight to analysis, a fractionation technique is often performed to partially purify the target compounds to improve subsequent analysis (Sarker et al., 2006). Separation of the crude extract into fractions based on polarity, acidity/alkalinity, charge or molecular weight usually involves various forms of column chromatography or solid phase extraction. After fractionation, the partially purified compounds are subject to some form of quantitative analysis, such as gas or liquid chromatography, commonly coupled with mass spectrometry (MS), FID (flame ionization detection) or nuclear magnetic resonance spectroscopy (NMR) (Sarker et al., 2006; Lee et al., 2017). Spectroscopic techniques may be used to aid in structure identification and verification of the compound, with the use of published articles. These techniques include ultraviolet spectroscopy, nuclear magnetic resonance, or infrared spectroscopy (Sarker et al., 2006). Crucial to quantitative analysis

is the inclusion of an authentic internal standard. The incorporation of an internal standard is important to gage accurate recovery estimates and correct for any variation (machine, sample or method wise). Overall, standard(s) used in quantitative analysis aid in calibrating detector responses and yield more accurate quantitative data.

Each step of an extraction method must be tailored to the natural product of interest, and subsequently, at each step, there should be testing and optimization. There are many challenges involved with extraction method development. Some of these are the compound(s) of interest may be retained on a column during the fractionation process, the time it takes during various steps (i.e. drying time, maceration time etc.), the compound(s) of interest may become unstable throughout the protocol, there may be interaction between the compound(s) of interest and other components in a crude extract that may alter precise separation and/or other issues with reproducibility. For example, Sporring et al., (2005) describes various extraction techniques that have been developed for the determination of polychlorinated biphenyls (PCB) in soil, and that multiple techniques can be performed to extract PCBs from soil, but with optimization, improvements can be made to existing protocols that lead to greater recoveries of PCB from soil samples.

The last step in developing an extraction method for a given type of compound is validation. Confidence in an extraction method is solidified through the inclusion of a validation test trial as this entails defining parameters such as the limit of detection, limit of quantification, equipment linearity, method specificity, accuracy (reproducibility) and precision (repeatability) (Leyva-Morales et al., 2015). The shift from non-specific or broad extraction methods to ones that focus on improving overall efficiency, reducing the amount of time it takes for extractions to be carried out and cost, without any compromise to extract quality, will yield an end product that is of greater research value.

Protocols for the extraction of ginsenosides from soil are inconsistent, with various authors reporting different methods. As identification techniques and methods are developed, new ginsenosides may be discovered. This is modeled by the fact that over time, the amount of new ginsenosides discovered from plant tissue and reported in

literature has increased to date (Qi et al., 2011; Chen et al., 2019). Similarly, ginsenoside isolation from plant tissue has increased substantially with improvements to purification and extraction protocols (Chen et al., 2019). Presently, studies that incorporate methods for the extraction of ginsenosides from soil involve air or oven drying samples, an extraction using methanol over a varied period of time, a solvent to soil ratio that is inconsistent amongst studies, followed by a filtration step. After this, the solvent is typically removed by rotary evaporation to dryness and the sample redissolved in various solvents and then analyzed using HPLC (Nicol et al., 2003; Yang et al., 2015; Li et al., 2019; Li et al., 2020). Previous unpublished data from the Bernards's lab suggest that ginsenoside extraction from soil is inconsistent. When using the typical, single overnight 80% MeOH solvent extraction, not all ginsenosides were recovered, and therefore more than one extraction may be required. Additionally, issues with reproducibility were apparent as field soils typically contained a high abundance of PPT but, in spiked soil samples trials, recovery of PPT was very poor. This highlights the need to improve the current protocol of extracting ginsenosides from soil. Undergoing a stepwise trial process to optimize the extraction of ginsenosides from soil could yield many positive insights. These include improving our understanding on the magnitude of the presence of these compounds and allow for a more accurate quantification and representation of these compounds in the soil.

Few studies to date, in the context of GRD, have extracted ginsenosides from soil. For instance, Yang et al., (2015) established the presence of ginsenosides in field soils and that these compounds exhibited autotoxic effects in emerging seedlings. However, these findings were based on *Panax notoginseng* (Chinese ginseng), not *Panax quinquefolius* (American ginseng). He et al., (2009) focused on the role of phenolic compounds, instead of ginsenosides, produced by American ginseng and their autotoxic effects on seedlings. These phenolic compounds were shown to reduce the growth of these seedlings. Furthermore, they were able to verify the presence of these compounds in the plow layer of the soil of commercially cultivated American ginseng fields in China. Li et al., (2020) focused on extraction of ginsenosides from soil spiked with a known composition of ginsenosides, based on field soils used to grow Chinese ginseng. Their aim was to determine if autotoxic ginsenosides from these soils were able to alter the soil fungal

microbiome. My project will provide novel insight in understanding and determining ginsenoside composition on Ontario, Canada field soil where American ginseng is grown, over time.

1.7 Thesis objectives

To truly capture the function of ginseng root exudates and their role in altering the soil ecology, ginsenoside composition and dynamics (accumulation and persistence over time) in soil must be established. This information will aid in establishing a link between ginsenosides and GRD, when coupled with data from microbiome analyses. However, with no reliable ginsenoside specific extraction method, characterizing the changes in soil chemistry is challenging. To accurately survey the soil for changes in ginsenoside composition, ginsenoside extraction protocol optimization and subsequent validation is required, and will help to characterize how ginsenoside composition changes over time. The objectives of this research project are to (i) develop, optimize and validate a protocol of extracting ginsenosides from soil, and (ii) apply the optimized protocol to isolate and quantify ginsenosides from ginseng garden soil, thereby tracking and how ginsenoside levels change in soil over time, focusing on their accumulation in newly planted gardens and persistence in recently harvested gardens.

Through the development of a robust ginsenoside extraction protocol, various hypotheses requiring soil ginsenoside analyses can be addressed. Through the development of this targeted extraction method, the aim is to produce a method that encompasses the following criteria and qualities: selective, sensitive, precise, consistent, and reproducible. Ultimately, this project will determine the changes in ginsenoside levels and composition and contribute to our understanding of the role these compounds play in GRD.

2 Materials and Methods

2.1 Soil collection

For the ginsenoside accumulation study, soil was collected from three independent commercial ginseng gardens (denoted site 1, 2, 3), located in Norfolk County, Southwestern Ontario (Figure 2.1). The gardens were seeded in August 2018, with germination occurring in Spring 2019. Soils were collected at nine different time points: Fall 2018, Spring 2019, twice in Summer 2019, Fall 2019, Spring 2020, twice in Summer 2020 and Fall 2020 (Table 2.1). None of the three sites had been used to grow ginseng in the past. For the ginsenoside persistence study, soil samples (provided by Dr. Oualid Ellouze, Agriculture and Agri-Food Canada, Vineland, Ontario) were collected from research garden plots at the Harrow Research Station (Harrow, Ontario) and Vineland Research Station (Vineland, Ontario) (Figure 2.2). Initial samples were obtained approximately one-month post-harvest (Fall 2018), and subsequently in the Summer of 2019 and Summer of 2020. The gardens were left un-disturbed post-harvest.

The samples were collected using a galvanized steel soil sampler (LaMotte). Two separate soil samplers were used; one to collect control soil (outside the ginseng garden) and the other to collect soil from within the ginseng gardens. For each site, five sampling areas within each ginseng garden were selected, and 3 cores (approximately 2.5 cm diameter, 30 cm deep) collected per area (n = 3 sites × 5 sampling areas). In site 1, the five sampling areas were selected by starting at the northeast edge of the field and going up the laneway to the 7^{th} bay, and in to the 5^{th} post. From there, soils were sampled from the middle bed in the bay at each successive post until 5 areas were sampled (Figure 2.3). At site 2, the five sampling areas were similarly selected by starting at the northwest edge of the field. At site 3, the five sampling areas were selected by starting on the southwest edge of the field. Control samples for all 3 sites were obtained by starting opposite the 7^{th} bay of each site, and sampling occurred every 1.5 posts along the edge of the field until 5 areas were sampled. Soil samplers were wiped clean using paper towel to remove debris between each of the 5 sampling areas. To avoid cross contamination between field sites, soil samplers were rinsed with 70% EtOH followed by DI H_2O, before leaving each site. Prior to entering each field, disposable field boot covers were worn to prevent field to

field contamination. After collection, the soil cores were kept frozen at -20ºC in plastic bags until being processed for extraction of ginsenosides. Prior to extraction, soils were oven dried at 55ºC to constant weight, and sieved with a 35 mesh (500 microns) (Fieldmaster ®) sieve to remove plant debris and large soil clumps.

Figure 2.1. Approximate locations of the commercial ginseng farms. Three sites were used to collect soil samples in Norfolk County, Southwestern Ontario, Canada (Google Maps, accessed August 2019).

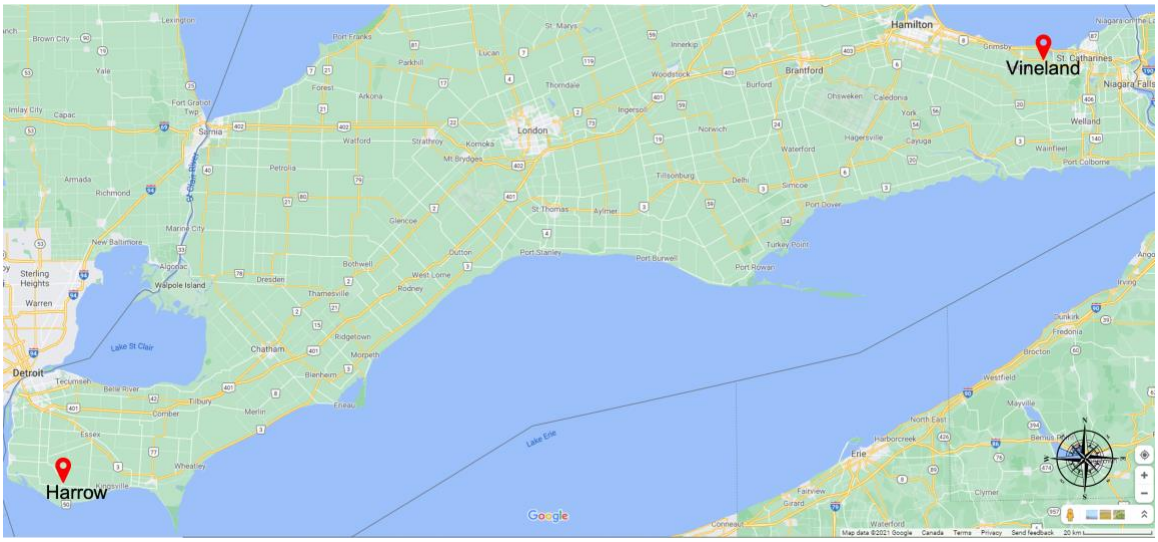

Figure 2.2. Location of persistence study research ginseng gardens. Two sites were used to evaluate persistence (Harrow Ontario and Vineland Ontario) (Google Maps, accessed September 2020).

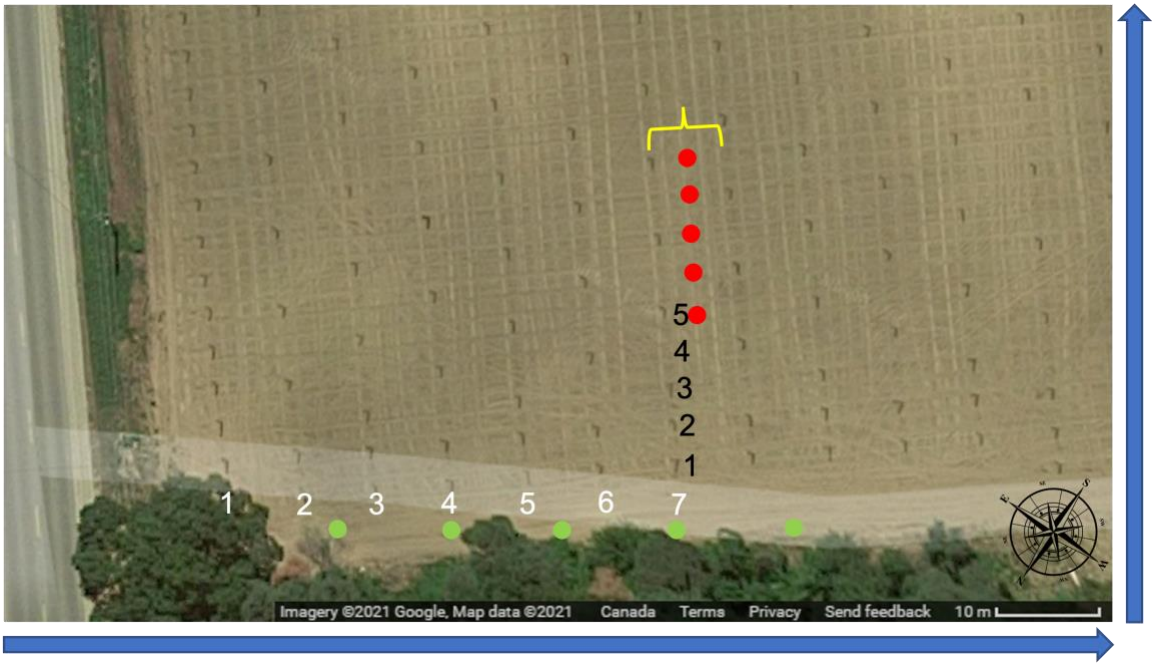

Figure 2.3. Example ginseng garden sampling location. Green dots indicated control locations (non ginseng garden) and red dots indicate experimental locations (within ginseng garden). White numbers indicate orientation to 7th bay, and black numbers indicate orientation within the field to the 5th post at which sampling occurred every post thereafter (Google Maps, accessed January 2020).

Table 2.1. Sample collection months and dates. Samples were collected 9 times throughout the duration of this project.

Month and year	Date
Fall 2018	October 23, 2018
Spring 2019	April 25, 2019
Summer A 2019	June 27, 2019
Summer B 2019	September 19, 2019
Fall 2019	October 29, 2019
Spring 2020	May 6, 2020
Summer A 2020	June 25, 2020
Summer B 2020	August 24 (Site 1, 3) and September 1, 2020 (Site 2)
Fall 2020	October 23 (Site 1, 3) and 25, 2020 (Site 2)

2.2 Soil composition

Bulk garden soil was collected from each site and used to determine soil type, composition and mineral content. Soil composition analysis was conducted at A & L Laboratories Inc. in London, Ontario Canada. Overall, the soil from each site was classified as loamy sand, with each site consisting of ~75-85% sand (Table 2.2; Supplementary Figure 1).

Table 2.2 Soil texture analysis conducted by A & L laboratories Inc.

Sites	Soil Analysis (%)			
	Sand	Silt	Clay	Textural class
Site 1	82.9	10.5	6.6	Loamy Sand
Site 2	76.9	14.5	8.6	Sandy Loam
Site 3	84.9	6.5	8.6	Loamy Sand

2.3 Soil spiking with ginsenosides

For extraction optimization, bulk control (non-garden) soil was spiked with a known amount of ginsenosides and used throughout the protocol optimization phases. Approximately 1100 g of wet soil from site 1 was dried in an oven for 7 days at 55°C. From this, 1000 g of dry soil was weighed, transferred to a bucket and spiked with 0.5 g of crude ginsenoside extract prepared from American ginseng roots, in 500 mL DI H_2O. The ginsenoside extract was slowly poured into the bucket with the dried control soil and mixed with a hand drill equipped with a 4" diameter paint mixer. The wet, spiked soil mixture was then dried for 7 days as above (indicative of the time at which soil was constant weight). After 7 days, the dried soil was re-mixed with the hand drill for another 5 minutes and dried for another 24 hours.

2.4 Ginsenoside extraction: Base protocol (pre-optimization)

To establish a baseline, ginsenosides were extracted from 20 g aliquots of dried soil using a single, 24 hour incubation with 80% MeOH (60 mL) on a gyratory shaker (adapted from Nicol et al., (2003)). All methanol (MeOH) used was analytical (LCMS) grade (Methanol, Optima™ HPLC, Fisher Chemical™). The samples were centrifuged at 1700 × g for 2 minutes at 21^{o}C to pellet the soil, and the supernatant transferred to a round-bottom flask. The soil pellet was washed once with an additional 60 ml 80% MeOH, and the extracts pooled. Extracts were dried *in vacuo*, and reconstituted in aqueous MeOH for LCMS analysis. To optimize this basic protocol, and improve the reproducibility of ginsenoside extraction from soil, the following parameters were examined:

- Number and duration of extractions
- Inclusion of solid phase extraction (SPE) clean-up step
- Ratio of solvent to soil
- Minimum amount of soil for analysis

These parameters were tested in sequential phases to establish optimal extraction conditions with the overall goal of producing the highest ginsenoside yield..

2.5 Number and durations of extractions

Extraction of 3 replicates of 20 g spiked soil was repeated three times (i.e. three 24 hour extractions over 3 days), with sequential extracts kept separate. After evaporating the extraction solvent and reconstituting the individual extracts in 25% MeOH, they were analyzed by LCMS (see section 2.13).

In a separate experiment (conducted after the soil threshold experiment below), spiked soil samples (5 g) were subject to 4 timed gyratory shaking trials with the goal of determining whether time spent on shaker could be decreased, as this would increase the overall efficiency of the protocol. Extractions were repeated three times (as above), except that the duration of each extraction was either 1, 4, 12 or 24 hours.

2.6 Solid phase extraction

For the SPE step, 3 mL, 200 mg bed weight, 40-60 µm particle, HyperSep™ C-18 solid phase extraction (SPE) columns (Thermo Scientific™) were used. To prepare them for use, columns were washed with 100% MeOH (3 mL) and equilibrated with DI H_2O (2 × 3 mL) to match the starting conditions of the extracts. Crude soil extracts were concentrated to aqueous (rather than dryness) and loaded directly onto the SPE columns. Column loading, washing and elution was facilitated by a vacuum manifold (Restek). After the samples were loaded, the columns were washed with 3 mL 30% MeOH, leaving the vacuum manifold on for ~1 minute for the bed to dry completely. Ginsenosides were eluted with four separate 1 mL volumes of 100% MeOH. Each Sep-Pak eluent (H_2O, 30% MeOH, 4 × 100% MeOH) was analyzed for ginsenosides. A full column volume (3 mL) of 100% MeOH (×1) followed by (3 mL) DI H_2O (×2) were applied to the column to prepare them for reuse or storage.

To ensure complete recovery of ginsenosides from SPE columns, three solvent tests were conducted. Test one involved following the above parameters with the final elution consisting of 4 × 1 mL 100% MeOH. Test two consisted of 4 × 1 mL of 100% HPLC grade acetonitrile as column eluent. Lastly, test three involved the final wash consisting of 4 × 1 mL of 100% MeOH and an additional 2 × 1 mL of acetonitrile. All eluents were

dried under N_2 using a 50 port RapidVap® Vertex Nitrogen Dry Evaporator with heating element set to 60ºC, and reconstituted in 25% MeOH for LCMS analysis.

2.7 Solvent to soil ratio

The optimal solvent to soil ratio was established by conducting extractions with varying solvent volumes. Twenty grams of spiked soil was used, and the following solvent volumes were tested: 60, 80 and 100 mL of 80% MeOH. Pooled extracts (3 × 24 hr) for each solvent-soil ratio were concentrated, processed through SPE columns and analyzed by LCMS.

2.8 Soil threshold

The minimum amount of soil required for extraction was determined by extracting decreasing amounts of spiked soil at a constant solvent volume to soil mass ratio. Based on the solvent to soil ratio experiment, a 4:1 ratio of solvent to soil was used. For this, the following amounts of spiked soils were extracted: 20 g soil + 80 mL solvent, 10 g soil + 40 mL solvent and 5 g soil + 20 mL solvent. Pooled extracts (3 × 24 hr) for each solvent-soil ratio were concentrated, processed through SPE columns and analyzed by LCMS.

2.9 Method limit of detection

The method limit of detection was determined by mixing control soils, which were collected from an area outside one garden site, with varying amounts of soil spiked with a known amount and composition of ginsenosides. The samples consisted of a series of soil samples of equal soil amount (5 g) but diminishing ginsenoside quantity. Spiked soil amounts were established based on expected ginsenoside values in these gardens. The spiked soil used was from a bulk mix that contained 0.02 g of crude ginsenoside extract into 40 g of control soil. The spiked soil series consisted of 6 dilutions, ranging from 0 g to 0.115 g of spiked soil, with the remaining soil equating to 5 g (Table 2.3) (n = 3). Based on this experimental data, the method limit of detection was determined, and the precent recovery of each ginsenoside established. These values provide a recovery efficiency that can be applied to field sample data.

Table 2.3. Series of spiked soil samples containing ginsenosides diluted with control soil.

Spiked soil (g)	Control soil (g)	Ginsenoside/sample (nmol)
0.00	5.00	0
0.007	4.993	5
0.014	4.986	10
0.029	4.971	25
0.058	4.942	50
0.115	4.885	100

2.10 Root ginsenoside profiles

Two-year old roots were collected from each of the 3 garden sites on October 23rd and 25th 2020. The roots from each site were kept separate in this analysis to establish whether root ginsenoside profiles differed between sites and how well they compared with respective soil ginsenoside profiles. The roots were initially stored at -20°C, and then dried in an oven for 12 days at 55°C. Taproot pieces were initially broken down using a hammer. Approximately 0.5 g of the dried broken root pieces were further pulverized using the hammer, and then ground in liquid N_2, with a mortar and pestle, to a fine powder.

Ground root tissue (20 mg) was added to 1 mL of 80% MeOH and placed on a rotating mixer for 48 hours. The extracts were centrifuged for 5 minutes at 12000 × g, then collected and transferred to a clean Eppendorf tube. For LCMS analysis, 50 µL aliquots were taken from each extract, dried using the 50 port RapidVap® Vertex Nitrogen Dry Evaporator with heating element and reconstituted in 50 µL of 25% MeOH containing 0.3125 ng/mL avenacoside-A (internal standard). Ginsenosides were analyzed using an established LCMS protocol (see section 2.13).

2.11 Internal standard selection

Three steroidal saponins were tested as potential internal standard candidates. Digitonin, derived from the foxglove plant (*Digitalis purpurea*), aescin, a saponin mixture from the horse chestnut (*Aesculus hippocastanum*) and avenacoside-A, derived from oats (*Avena sativa*). Several properties were considered when choosing the internal standard, including structural similarity to ginsenosides, absence from field soils, and distinct signals in the chromatogram for ease of identification and peak clarity. Each compound was analyzed individually by LCMS to determine whether signals were clear and identifiable. Avenacoside-A, a glycosylated steroid saponin (Osbourn, 1996), was the optimal internal standard. For chromatogram peak normalization, avenacoside-A was added (final concentration 0.3125 µg/mL) to samples when they are reconstituted after the SPE step in the extraction protocol.

Internal standard concentration was initially determined by analyzing a 1:1 dilution series (up to 12 dilutions starting with 1 mg/mL) to determine an appropriate target concentration. For field sample analysis a 15 mL bulk avenacoside-A solution was created with a final concentration of 0.6250 µg/mL in H_2O. This was added in equal volume to each ginsenoside sample to yield a final avenacoside concentration of 0.3125 µg/mL.

2.12 Method validation

The final, optimized protocol was subject to a validation test to ensure rigour. The optimized protocol included the following parameters (Figure 2.4):

- 5 g spiked soil
- 4:1 solvent – soil ratio (v/mass)
- 2 × 1 hour extraction, followed by a third overnight extraction (minimum 18 hrs)
- SPE column clean-up (4 × 1 mL MeOH elutions, pooled)
- Reconstitution in 25% MeOH containing 0.3125 µg/mL avenacoside-A

For method validation, soils were spiked with known amounts of ginsenosides, but with varying composition (Table 2.4). The amounts of each ginsenoside standard in each

solution were chosen to mimic biologically relevant concentrations found in American ginseng roots. Altogether, there were 4 spiked solution combinations used in this trial, consisting of a control (water only, no ginsenosides), a solution containing all the ginsenosides, a solution consisting of the diols only, and finally a solution consisting of the triols only.

Table 2.4. Amount of each ginsenoside (mg) in four of the mixes (per 5 g of soil) for the validation trial.

Ginsenosides		Control	All Ginsenosides	Diols only	Triols only
Protopanaxadiols	Rb1	0	0.70	0.70	0
	Rc	0	0.14	0.14	0
	Rb2	0	0.14	0.14	0
	Rd	0	0.42	0.42	0
	Gypenoside XVII	0	0.42	0.42	0
	F2	0	0.42	0.14	0
Protopanaxatriols	F11	0	0.42	0	0.42
	Rg1	0	0.14	0	0.14
	Re	0	0.56	0	0.56

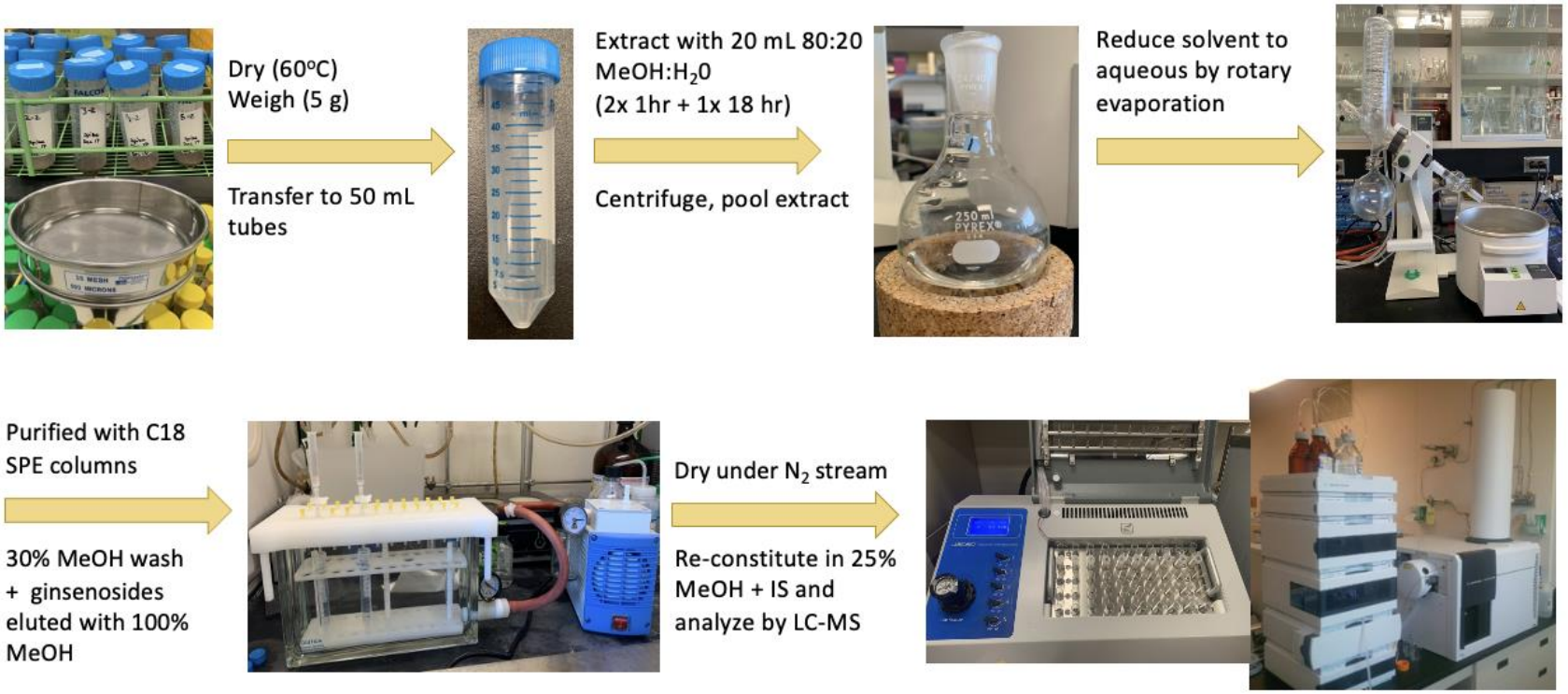

Figure 2.4. Experimental design schematic for ginsenoside soil extraction.

2.13 Ginsenoside analysis by LCMS

Liquid chromatography mass spectrometry (LCMS) (1260 LC coupled to 6230 TOF MS, Agilent Technologies) was used to identify and quantify the ginsenosides in the spiked soil experiments and field soil extracts. The analysis method was adapted from Ivanov et al. (2016). Samples (2μL) were injected onto a Zorbax Extend C-8 column (2.1 × 50 mm, 1.8 mm, Agilent Technologies, Santa Clara, CA, USA), and eluted with a gradient of CH_3CN (Solvent B: 90% CH_3CN in H_2O containing 0.1% HCO_2H and 1 mg/L NaOAc) in H_2O (Solvent A: containing 0.1% HCO_2H and 1 mg/L NaOAc) as follows: Initial conditions 20% B in A, held for 1 min, followed by a linear gradient to 35% B over 2 min, and 100% B over 6 min, and held at 100% B for 1 minute before returning to start conditions. The column was equilibrated at 20% B for 10 minutes between samples. Cumulative sample run time was 23 minutes. The flow rate was set to 0.40 mL/min, and the eluent monitored at 203 nm before infusion into the mass spectrometer through a Dual Spray ESI (electrospray ionization) source with gas temperature of 300°C flowing at 12 L/min, and a nebulizer pressure of 45 psi. The fragmentor voltage was set to 120 V with a Vcap of 4500 V. Automated internal calibration was done using reference ions 121.0508 and 922.0096 *m/z*. Ginsenosides were detected and quantified as their Na-adducts, in positive ion mode $[M + Na]^+$ (Table 2.5).

Table 2.5. Mass accuracy of quant ions used for quantification of ginsenosides. Ginsenoside-specific quant ions (mass signals (*m/z*) consistent with ginsenoside compounds as their Na+ adducts), were used to generate and integrate extracted ion chromatograms from total ion chromatograms. Mass accuracy was calculated using the average mass for each ginsenoside found in a mixture of ginsenoside standards analyzed alongside each batch of field samples (N = 9).

Ginsenosides	Theoretical Exact Mass	Average Mass Found $[M+Na]^+$	Mass Found (Std. Dev.)	Mass Accuracy (ppm)
Rb1	1131.5922	1131.5897	± 0.003	± 2.24
Rc	1101.5816	1101.5807	± 0.006	± 0.79
Rb2	1101.5816	1101.5805	± 0.005	± 1.03
Rd	969.5393	969.5398	± 0.006	± 0.46
Gypenoside XVII	969.5393	969.5391	± 0.005	± 0.17
F2	807.4865	807.4859	± 0.004	± 0.70
F11*	801.5051	801.5004	± 0.004	± 5.83
Rg1	823.4814	823.4823	± 0.003	± 1.05
Re	969.5393	969.5399	± 0.004	± 0.61

* Ginsenoside F11 mass detected without Na^{+-}

2.14 LCMS calibration

Ginsenoside standards: Rb1, Rb2, Rb3, Rc, Rd, Gypenoside XVII, F2, F11, Rg1 and Re (Chengdu Biopurify) were used to generate calibration curves. To produce the calibration curves for the ginsenoside standards, a range of serial dilutions were created using 50% MeOH. The concentration for each ginsenoside standard ranged from 0.03-62.5 mg/mL. The standards were spiked with avenacoside-A at a concentration of 0.0128 mg/ml. Each concentration was analyzed in triplicate and the calibration curves were plotted as a function of peak area by concentration. Linear regression analysis yielded straight line (y = mx+b) calibration equations (Table 2.6).

The machine limit of detection (LOD) was determined using data from the calibration experiment. The LOD is important for determining the lowest quantity or concentration of the compound of interest that can be detected with reliability within a stated analytical

method (Boqué and Heyden, 2009). For each ginsenoside, a LOD value was generated from the following equation LOD = 3.3σ, where σ is the standard deviation of the mean of the lowest detectable concentration of standard (Boqué and Heyden, 2009).

Table 2.6. Calibration curves and LOD for common ginsenosides monitored in this study.

Ginsenoside	Calibration curves	R^2 value	LOD (pmol/µL)
Rb1	Y = 39581x - 1351	0.9959	0.008
Rb2	Y = 25131x - 3875	0.9994	0.018
Rc	Y = 21183x - 4117	0.9979	0.001
Rd	Y = 20303x - 912.2	0.9993	0.013
Gypenoside XVII	Y = 28697x - 9423	0.9939	0.024
F2	Y = 30077x - 3.829	0.9997	0.002
F11	Y = 28062x - 4014	0.9965	0.049
Rg1	Y = 26139x - 2070	0.9997	0.008
Re	Y = 25967x - 2488	0.9999	0.016

2.15 Final optimized protocol

Combining the above tests produced the optimized protocol. The summarized protocol is described as follows. The protocol begins with drying soil to constant dryness (for 7 days). Once dried, the samples were sieved using a 35 mesh (500 microns) (Fieldmaster ®) sieve to remove plant debris and large soil clumps, and 5 g of dried soil was weighed out into 50 mL Falcon™ tubes. Then, 20 mL of 80% MeOH was added to each sample and placed on a gyratory shaker at 175 rpm. Extracts were collected 3 times; in two consecutive 1 hour extractions followed by an overnight extraction lasting a minimum of 18 hours. For each extract collection, the samples were centrifuged at 1700 × g for 5 minutes to pellet the soil, and the extracts were collected. Extracts were pooled, transferred to round bottom flasks (either 100 mL or 250 mL) and rotary evaporated to aqueous state. Samples were transferred from the round bottom flasks to 15 mL Falcon™ tubes. The samples were loaded onto water-equilibrated HyperSep™ C-18 solid phase extraction (SPE) columns (Thermo Scientific™). To equilibrate the SPE columns, they

were first washed with 1 column volume (3 mL) of 100% MeOH, and then equilibrated with 2 column volumes (6 mL total) of DI H_2O. Samples were loaded onto the columns and then washed with 30% MeOH (3 mL). Ginsenosides were eluted with 100% MeOH (4 × 1 mL) directly into 15 mL Falcon™ tubes. Column loading, washing and elution was facilitated by a vacuum manifold (Restek). Ginsenoside eluents were incrementally transferred to Eppendorf tubes and dried down under N_2 using a 50 port RapidVap® Vertex Nitrogen Dry Evaporator at 60ºC. Finally, the samples were reconstituted with 25 μL of 50% MeOH, and diluted with 25 μL of avenacoside-A (0.625 ng/mL in DI H_2O), containing 0.3125 ng/mL avenacoside-A (internal standard). The samples were then run and analyzed via LCMS.

2.16 Data analysis

Data were analyzed using MassHunter Workstation Qualitative Analysis Software (version B.05.00) (Agilent Technologies Inc. 2011®). Data files were loaded into the qualitative analysis software module. Extracted ion chromatograms were derived from total ion chromatograms (Figure 8), by inputting targeted *m/z* values of interest (ginsenoside specific; Table 5). Quant ion *m/z* were set to symmetric (*m/z*) ± 0.1000 (tolerance). Extracted ion chromatograms were integrated after extraction (Figure 8). From this output, peak areas, retention times and *m/z* values were recorded.

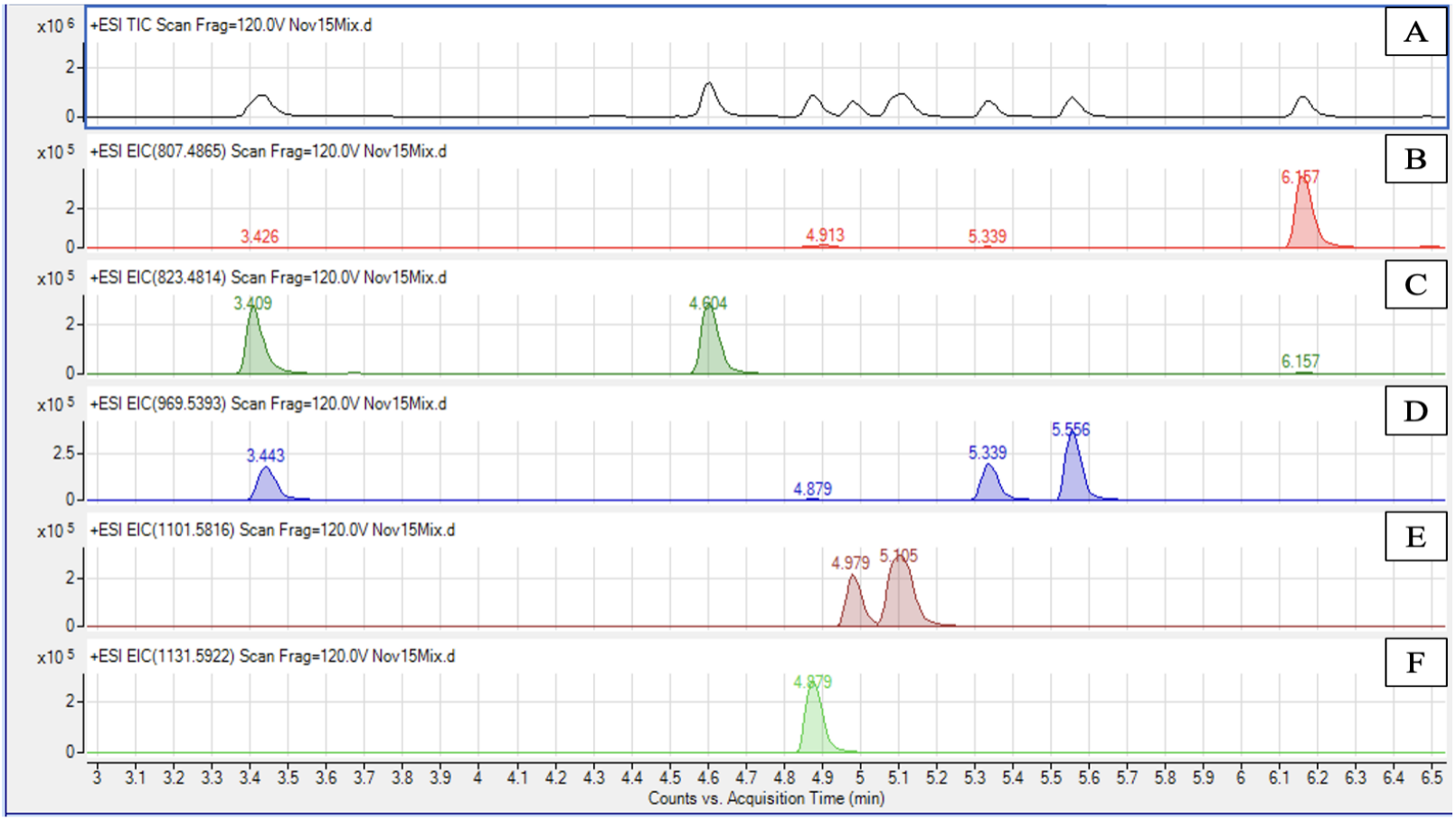

Figure 2.5. LCMS analysis of purified ginsenoside standards. A, Total Ion Chromatogram (TIC) for a mixture of ginsenoside standards. B-F, Extracted Ion Chromatograms (EIC) generated from the TIC, using theoretical exact mass. B, F2 (RT 6.167) at *m/z* 807.4865. C, Rg1 (RT 3.409) and F11 (RT 4.604) at *m/z* 823.4814. D, Re (RT 3.443), Rd (RT 5.339) and Gypenoside XVII (RT 5.556) at *m/z* 969.5393. E, = Rc (RT 4.979) and Rb2/3 (RT 5.105) at *m/z* 1101.5816. F, Rb1 (RT 4.879) at *m/z* 1131.5922. RT = retention time (minutes).

2.17 Statistical analysis

For field data, a general liner model and ANOVA using RStudio (version 1.1.456) was used to compare the quantity of ginsenosides in each sample collected (control and garden) over the sampling period. To reduce the skew in the field data, the values were log transformed to normalize the data set. Where significant differences were found, a Tukey's HSD post hoc test was used to compare total ginsenoside content in each sample and identify where the differences occur (pairwise differences). The treatments pertain to the source of soil (garden soil and control soil, respectively), with the factor being time. Statistically significant differences were determined using $p < 0.05$. Statistics for field data were run in RStudio using the package stats (RStudio team, 2021, R Core Team, 2021).

3 Results and Discussion

3.1 Protocol optimization

3.1.1 Number of extractions and solid phase extraction

The C-18 SEP paks were used to clean the samples prior to chromatographic analysis. Analysis output from the LCMS established that the H_2O and 30% MeOH washes did not contain any ginsenosides (Table 3.1). Furthermore, washing the sample with 2 × 1 mL of 100% MeOH was not sufficient to recover all ginsenosides (Table 3.1). With two column washes of 100% MeOH, trace amounts of ginsenosides were present, thus to ensure as much as possible were removed from a column, four column washes of 100% MeOH were deemed sufficient (from a time and efficiency perspective) to remove the bulk of the ginsenosides from the C-18 columns. The number of extractions was limited to three as the third extract analyzed yielded only trace amounts of ginsenoside (Table 3.1), as subsequent column washes yield less and less ginsenosides.

To further ensure 100% MeOH was an optimal elution solvent, tests were conducted using 4 × 1mL 100% MeOH, 4 × 1mL 100% acetonitrile, and 4 × 1mL 100% MeOH + 2 × 1 mL 100% acetonitrile. The use of 100% MeOH consistently showed the greatest ginsenoside yield, compared to any test that used acetonitrile (Supplementary table 1). Based on this, 100% MeOH was used as the elution solvent.

Ginsenosides are large, amphipathic molecules, and as the extract runs through the SEP column, they partition into the C-18 column matrix. After loading the extracts onto the columns under aqueous conditions, the addition of a 30% MeOH wash assisted in removing non target molecules from the extracts, while allowing the ginsenosides to remain in the stationary phase of the C-18 column. With the final addition of 100% MeOH, the ginsenosides preferentially move from the stationary phase through the mobile phase and are collected then analyzed for quantification via LCMS analysis. Overall, the inclusion of the C-18 column step helps to concentrate the ginsenosides, and also clean them of any non-relevant molecules (reduce ion suppression), prior to chromatographic analysis.

Table 3.1. Peak area of ginsenosides in sequential extractions. Data are for each of three consecutive extractions of the same soil sample, and the subsequent washes (H_2O, 30% MeOH) and elution (100% MeOH - 1, 100% MeOH - 2) from SPE columns (N=1).

Ginsenosides	Extraction #1				Extraction #2				Extraction #3			
	H_2O	30	100 {1}	100 {2}	H_2O	30	100 {1}	100 {2}	H_2O	30	100 {1}	100 {2}
	(Peak area)				(Peak area)				(Peak area)			
Rb1	0	0	401414	1010358	0	0	833140	57578	0	0	226353	18424
Rb2	0	0	64616	7683	0	0	70719	0	0	0	8459	0
Rc	0	0	50061	138525	0	0	120892	9171	0	0	22402	1363
Rd	0	0	187892	488943	0	0	393214	34843	0	0	93973	8348
Gypenoside XVII	0	0	190488	236285	0	0	239905	9188	0	0	31360	1878
F2	0	0	54964	90098	0	0	67567	7898	0	0	7993	1316
Rg1	0	0	114109	166111	0	0	322227	3012	0	0	74700	511
Re	0	0	1590951	776754	0	0	1316765	24117	0	0	301948	8336

3.1.2 Duration of extractions

To determine the duration of the three extractions, a timed trial was conducted to solely evaluate the optimal time for extractions that would yield the most ginsenosides. For each time point, three separate extractions were collected and analyzed to determine which would produce the most efficient combination of time and highest ginsenoside yield. There was minimal difference in the amounts of ginsenosides present in the 1 hour and 4 hour extraction across all 3 extracts (Table 3.2). Furthermore, that same pattern remained for the 4 and 12 hour extraction, such that the peak areas were relatively consistent for those respective extraction times (Table 3.2). The 24 hour extraction produced similar peak areas for extract 1 and 2, compared to the same extracts for the 1, 4, and 12 hour extraction times (Table 3.2). The third extract in the 24 hour test resulted in the lowest ginsenoside peak areas (Table 3.2). Factoring in protocol efficiency and time management, the optimal combination of extractions was the following: 1 hour + 1 hour + overnight (~18-24 hours). This combination of extraction times would allow researchers to conduct their experiments within a reasonable working day and time frame while being able to largely extract as much of the compounds out of the soil as possible. Overall, the duration of the extraction procedure was determined by conducting three extractions over a single ~24 hour period, which creates a significant improvement to time for researchers who use this method. To date, no other published research has conducted trials looking at extraction time of ginsenosides from soil. The limited research available report ginsenoside extraction from soil to consist of a single 24 hour extraction (Nicol et al., 2003; Yang et al., 2015),

Table 3.2. Peak areas of ginsenosides in separate extracts over 4 different time periods. Collections occurred three times every 1, 4, 12 and 24 hours (n = 3).

Ginsenosides	1 hour			4 hour		
	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3
	(Peak area)			(Peak area)		
Rb1	36487	10747	5056	32462	9693	1331
Rb2	2463	1360	1050	2129	1113	278
Rc	2744	419	0	2390	0	0
Rd	9376	121898	11000	9112	10310	3491
Gyp. XVII	1626	460	926	2057	671	0
F2	1478	1987	2485	1940	163947	0
Rg1	1836880	932775	481491	1638425	1000956	381663
Re	10169	2132	1586	9349	2235	659
Ginsenosides	12 hour			24 hour		
	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3
	(Peak area)			(Peak area)		
Rb1	37611	8978	1152	32243	13669	0
Rb2	3328	1050	233	2569	1946	240
Rc	2902	110	0	2460	444	0
Rd	12525	11664	2567	9622	14046	1481
Gyp. XVII	1963	643	0	2103	1398	0
F2	1985	1681	3040	2086	1940	0
Rg1	1521568	720919	140996	1517079	1061979	295691
Re	9741	2102	788	9027	2823	1042

3.1.3 Solvent to soil ratio

A combination of soil to solvent ratios was tested to determine the optimal amount of solvent for extraction. The following combinations were tested: 20 g + 60 mL, 20 g + 80 mL and 20 g + 100 mL of 80% MeOH. Using 60 mL of solvent resulted in a total ginsenoside peak area of 9.5 million, 80 mL of solvent produced a total peak area value of 10.1 million and finally using 100 mL of solvent produced a total peak area value of 9.3 million (Table 3.3). Factoring in product cost, using 80 mL of solvent was deemed most appropriate from a ginsenoside yield and cost perspective. The data from this test established the optimal volume of soil to solvent for the protocol (1:4). Nicol et al., (2003) and Yang et al., (2015) both conducted ginsenoside extractions from soil, and each use 80% MeOH. Nicol et al., (2003) do not report soil amount and volume of solvent used, and Yang et al., (2015), report a 1:3 ratio of soil to solvent.

Table 3.3. Peak areas of ginsenosides from extractions using 20 g of soil and 60 mL, 80 mL or 100 mL of 80% MeOH. For each solvent volume, four SEP pak column elutions were collected and analyzed individually.

Ginsenosides	60 mL			
	1	2	3	4
	(Peak area)			
Rb1	953012	603521	105796	26065
Rb2	387771	136639	20207	4198
Rc	243044	255034	39424	8342
Rd	1416133	1137309	368868	74690
Gypenoside XVII	799446	488710	65113	12038
F2	751908	795565	145697	27640
Rg1	454218	43680	0	0
Re	162140	17598	0	0
TOTAL	9543806			

Table 3.3. Continued

Ginsenosides	80 mL			
	1	2	3	4
	(Peak area)			
Rb1	1053828	492547	71255	17240
Rb2	379046	108300	13870	2858
Rc	258039	208829	24746	4809
Rd	1458649	1081262	235787	42166
Gypenoside XVII	804078	400766	42178	8182
F2	737940	663086	82271	16384
Rg1	471619	28499	0	0
Re	1458649	0	0	0
TOTAL	10166883			
Ginsenosides	100 mL			
	1	2	3	4
	(Peak area)			
Rb1	1060752	650469	66148	16636
Rb2	377310	146374	13241	3173
Rc	291970	279426	21321	4584
Rd	1348122	1159117	221609	42119
Gypenoside XVII	833479	512497	39649	7818
F2	748583	741648	83733	17437
Rg1	447188	54038	0	0
Re	126847	17208	0	0
TOTAL	9332496			

3.1.4 Soil threshold

Using the outcome from the solvent-to-soil ratio experiment, a test was conducted to determine the minimum amount of soil required in which ginsenosides could be detected, while keeping a consistent soil to solvent ratio (1:4). The soil threshold that was consistent with a 1:4 ratio of soil to solvent, yielding the smallest and minimum mass

with the greatest yield would be selected. The following combinations were tested: 5 g soil + 20 mL solvent, 10 g soil + 40 mL solvent, and 20 g soil + 80 mL of solvent. Three separate replicate tests confirmed that using 5 g of soil with 20 mL of solvent produced the highest yield of ginsenoside (7.2 million peak area) (Table 3.4). Testing soil threshold was important in terms of soil availability (i.e., how much sample had to be collected for analysis) and protocol efficiency. The commercial garden soil samples are finite, such that only roughly 60 g of soil per area (with 5 sampling areas total) on a ginseng bed, from each of the three ginseng gardens were collected. Other authors have reported 20 g up to 500 g of soil used for ginsenosides extraction (Nicol et al., 2003; Yang et al., 2015). Improvements in sample allowance are important for conserving remaining garden soil for future analytical or molecular analysis. Furthermore, using 5 g compared to greater amounts of soil displayed higher ginsenoside yield.

Table 3.4. Ginsenoside yield (peak area) for three different soil threshold extractions aligning with a 1:4 ratio of soil to solvent.

Replicate	Total Ginsenoside (peak area)		
	5 g	10 g	20 g
1	4848609	610323	57945
2	5143433	2374468	3926481
3	11809279	2709342	2470608
Average	7267107	1898044	2151678

3.1.5 Method limit of detection

The method limit of detection experiment was conducted to determine the how sensitive this protocol was in detecting ginsenosides. Soils were collected from a control area from one of the gardens and spiked with a known amount and composition of ginsenosides. This experiment was designed to create dilution series of soils, by diluting spiked soil with increasing amounts of un-spiked soil. These samples were of equal soil amount but with decreasing amounts of ginsenosides (of known quantities). The method limit of detection for each ginsenoside ranged from <5 to <50 nmol ginsenoside/g of soil (n = 15) (Table 3.5).

On an individual ginsenoside level, the average % recovery was not uniform and ranged from 2-113%. What was consistent however, was the higher recovery of the PPT relative to the PPD. The PPD had percent recoveries that ranged from 2-19% whereas the PPT displayed greater percent recoveries that ranged from 25-113%. Notwithstanding the wide variation in individual ginsenoside recovery, across all compounds, only 15% of ginsenosides were recovered (Table 3.6). These data indicate that there are still inconsistencies with recovering ginsenosides from soil on an individual level. In a study conducted by Corbit et al., (2005), four different extraction methods and their respective recoveries of six ginsenosides were compared. Ginsenosides were extracted from ginseng roots directly, which poses a more direct and streamline extraction efficiency compared to soil. Despite this, of the 6 target ginsenosides, the % recovery ranged from 62-100%. Extracting plant natural products like ginsenosides from a material that is not derived from the source itself i.e., soil vs root or leaf, can lead to high % recovery variability. It is thought that factors such as soil composition, drying procedures, extraction duration and number, solvent concentrations etc., can lead to possible losses of these compounds throughout different stages of the protocol procedure, and lead to a lower % recovery (Sarker et al., 2006). Further experimental trials and analysis into individual ginsenoside recovery could be conducted to improve these inconsistencies.

Table 3.5. Recovery (mean ± SD) of ginsenosides from spiked soil samples (n = 15).

Ginsenoside		Ginsenoside Recovery	
		%	LOD (nmol/g soil)
Protopanaxadiols	Rb1	11.2 ± 0.03	<0.17
	Rc	19.3 ± 0.06	<0.03
	Rb2	3.9 ± 0.02	<0.14
	Rd	17.1 ± 0.05	<0.42
	Gypenoside XVII	2.0 ± 0.01	<0.10
	F2	2.3 ± 0.01	<0.01
Protopanaxatriols	F11	24.9 ± 0.12	<2.41
	Rg1	65.6 ± 0.30	<1.17
	Re	113.6 ± 0.59	<3.57

Table 3.6. Ginsenoside loads (nmol/g) in spiked soil samples, compared to ginsenoside loads recovered from soil (nmol/g). The average % recovery reported as ginsenoside load (mean ± SD).

Sample	Total ginsenoside load					
Total ginsenosides in spiked soil (nmol/g)	0	0.9	1.7	4.3	8.6	17.2
Ginsenosides recovered from soil (nmol/g)	0	0.1	0.1	0.9	1.5	3.4
% Recovery	0	9.7	8.1	20.3	17.2	19.6
Average % recovery (Std. Dev.)	15.0 (0.06)					

3.1.6 Method validation

The above parameters were collated into a revised protocol and subject to a validation experiment to ensure protocol rigour and confirm that the correct composition of ginsenosides were recovered from spiked soils. The optimized protocol was applied to a series of spiked samples of differing ginsenoside composition. When soils were spiked with the water only, no ginsenosides were present apart from trace amounts of Rb1 (Table 3.7). To recall, bulk control soil was taken from the perimeter of the ginseng gardens. It is possible the perimeter of the fields contained trace amounts of ginsenosides, resulting in the presence of Rb1 in these control soils. When the soil was spiked with a mix containing all the ginsenosides, post extraction, all the compounds were detected (Table 3.7). It was also evident that the PPD were recovered in lower amounts than the PPT. When spiking soils with a mix containing diols only, post extraction, only PPD were detected (Table 3.7). In a mix containing PPT only, post extraction, PPT were detected as well as trace amounts of PPD. This highlights that unexplained trace amounts are present (origin unknown), despite efforts to reduce this occurrence (i.e., use of new SPE columns for each sample to limit cross contamination). Additionally, the possibility of cross contamination exists as well. However, neither proposed option has a known explanation to date. Consistent with the method limit of detection experiment, the percent recoveries on an individual ginsenoside level were also highly variable. Possibilities for this could be that the compounds may be irreversibly bound to the soil matrix initially or lost or degraded during the extraction protocol. A deeper analysis into ginsenoside

breakdown products may shed light on the inconsistencies that occur and with the variable percent recoveries.

The PPT Re, Rg1 and F11 are structurally distinct to that of the PPD Rb1, Rc, Rb2, Rd and Gypenoside XVII. It is likely not the case for the PPD to breakdown or convert to yield a PPT product. This is simply because there is no vector to allow for the addition of a new hydroxyl group and the glycosylation at C-6 of a PPD to form a PPT throughout this protocol. Microbial interaction with the compounds may occur to produce this outcome; however, in this protocol the soil is pre-dried to a temperature that prevents the growth of microorganisms and extracts are in organic solvent. Only within each class can conversions occur. More specifically, the sugar side chains may be lost to yield a different ginsenoside. An example of this would be if ginsenoside Rb1 loses one unit sugar from the C-20 position, it would yield the ginsenoside Rd. Following this, if Rb1 lost one sugar from the C-3 position, it would produce Gypenoside XVII. Similarly, if Gypenoside XVII lost one sugar at the C-20 position, that would yield ginsenoside F2. For the PPT, a loss of a sugar from the C-6 position on Re would result in Rg1. F11 is considered a part of the PPT class, but is structurally distinct from Re and Rg1. A combination of these transformations during the extraction protocol may explain the varying levels of percent recoveries within a class. Investigation into why PPT are consistently recovered with higher efficiency compared to the PPD will improve reproducibility and reduce variability however, at the moment, the underlying mechanism is unknown.

Table 3.7. Recoveries of ginsenosides from each of the four spiking solutions (N = 3). "TR" indicates trace presence of compound post extraction, but not in spiked solutions.

Ginsenosides		Mix 1 Control	Mix 2 All Ginsenosides	Mix 3 Diols Only	Mix 4 Triols Only
Protopanaxadiols	Rb1	TR	64.7	36.4	TR
	Rc	0.0	69.2	370.0	TR
	Rb2	0.0	0.0	0.0	TR
	Rd	0.0	38.7	26.2	0.0
	Gypenoside XVII	0.0	63.3	33.6	0.0
	F2	0.0	50.3	24.1	TR
Protopanaxatriols	F11	0.0	88.0	0.0	61.4
	Rg1	0.0	110.5	0.0	77.5
	Re	0.0	85.2	0.0	77.9

3.1.7 Rb1 spiking trial analysis

Rb1 is the most abundant ginsenoside found in American ginseng. It is often regarded as the parent ginsenoside, because its core structure, if deglycosylated or broken down, can yield other ginsenosides. This spiking test was conducted to determine whether soils spiked with just Rb1 and processed using the optimized protocol would yield a variety of ginsenosides due to Rb1 breakdown during extraction and processing. Soil samples were spiked with 3 different concentrations of Rb1. For one set of samples 5 g of non-spiked soil (n = 3) was weighed into falcon tubes and each tube was spiked with a solution containing 0.10 mg of Rb1. A second set of samples (n = 3), each with 5 g of non-spiked soil was spiked with a solution containing 0.20 mg of Rb1. Finally, a set of samples (n = 3) was spiked with a solution containing 0.40 mg of Rb1. The samples were left to air dry for 3 days and were subsequently processed following the optimized protocol.

The method limit of detection and validation experiments highlighted inconsistencies in the percent recovery of individual ginsenosides. To understand some of these inconsistencies and shed light on possible ginsenoside conversion and transformation,

soils were spiked with varying amounts of Rb1. Rb1 can be considered the parent PPD ginsenoside, as the removal of sugars from various positions on the carbon skeleton can yield other ginsenosides. It is also the most abundant ginsenoside found in roots (Lim et al., 2005). After spiking soils with solutions that contained only Rb1, trace amounts of other ginsenosides (esp. Rd, Gyp XVII and F2) were also found in subsequent extracts (Table 3.8). The ginsenosides found in trace amounts could be derived from Rb1 via deglycosylation during the extraction protocol. This is shown schematically in Figure 3.1; if Rb1 loses a sugar at the C-20 position, that would yield the ginsenoside Rd. Additionally, a loss of a sugar at the C-3 position on Rb1 would produce Gypenoside XVII. Similarly, if Rb1 lost one sugar at the C-20 position and one sugar at the C-3 position, that would yield ginsenoside F2. These transformations are logical explanations for the presence of Rd, Gyp XVII and F2 in extracts from soils spiked with Rd; however, the exact mechanism that explains the transformations precisely is not yet known.

Table 3.8. Percent recoveries of ginsenosides from each of the three Rb1 spiking solutions (n = 3). "TR" indicates trace presence of compound post extraction, but not in spiked solutions.

Ginsenosides	Rb1 (mg)		
	0.1	0.2	0.4
Rb1	67.9	76.3	64.4
Rb2	0	0	0
Rb3	0	0	0
Rc	0	0	0
Rd	0	TR	TR
Gypenoside XVII	TR	TR	TR
F2	TR	TR	TR
F11	0	0	0
Rg1	0	0	0
Re	0	0	0

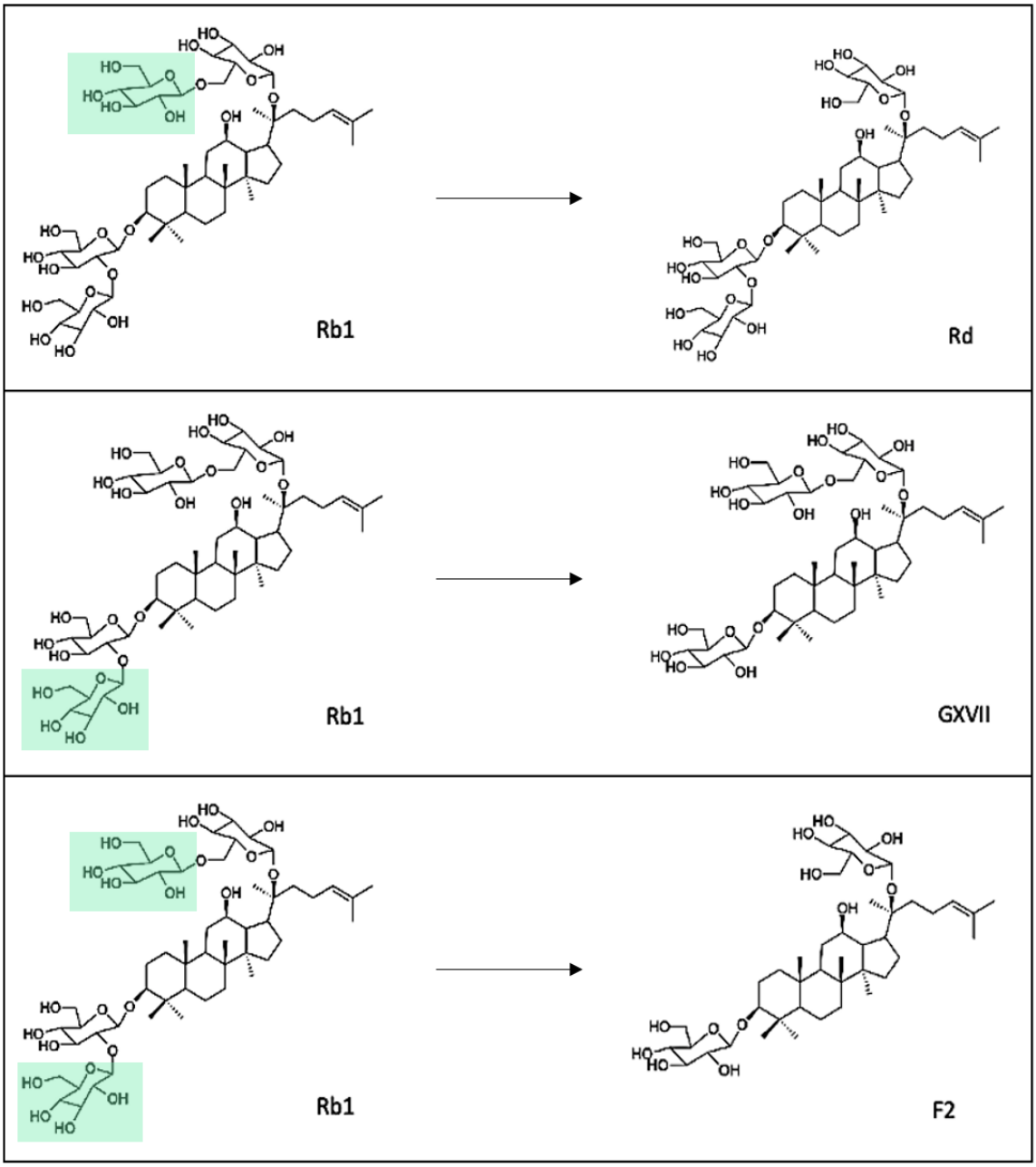

Figure 3.1. Parent ginsenoside Rb1 and its potential breakdown products. Green shading indicates the target sugar that is removed to yield a different ginsenoside.

3.1.8 Original and optimized protocol

As an additional measure to gage protocol improvement, the original protocol was directly compared to the optimized protocol. The original protocol involved a single 24 hour extraction using 20 g of spiked soil and 40 mL of 80% MeOH, followed by re-

extraction for 1 minute using a Vortex-Genie® (Scientific Industries). The optimized protocol involved two consecutive 1 hour extractions followed by a single 20 hour extraction, using 5 g of soil and 20 mL of 80% MeOH. In both protocols, the samples were centrifuged at 1700 × g for 5 minutes to pellet the soil, and the extracts were collected and pooled together (n = 3 for each extraction).

Before rotary evaporating, the original protocol extracts were filtered using No. 1 Whatman paper and collected in 15 mL Falcon™ tubes to store, before being added incrementally to 25 mL round bottom flasks for rotary evaporation. In the optimized protocol the collected extracts were directly added to 100 or 250 mL round bottom flasks. In the original protocol the solvent was evaporated by rotary evaporation to complete dryness, whereas in the optimized protocol, the sample was evaporated to aqueous, saving a considerable amount of time. In the original protocol, the dried compounds were quantitatively transferred by the addition 0.5 mL of 100% MeOH, repeated twice totaling 1 mL. In the optimized protocol, the aqueous extracts were passed through a HyperSep™ C-18 solid phase extraction (SPE) columns (Thermo Scientific™). The columns were washed with 1 column volume (3 mL) of 100% MeOH, and then equilibrated with 2 column volumes (6 mL total) of DI H_2O. The aqueous extracts were then loaded onto the column, followed by a 30% MeOH rinse, and finally the extracts were eluted with 100 % MeOH (4 × 1 mL) and collected in 15 mL Falcon™ tubes. In both protocols, the extracts were transferred to Eppendorf tubes to be dried down under N_2 using a 50 port RapidVap® Vertex Nitrogen Dry Evaporator with heating element and reconstituted in 25% MeOH for LCMS analysis.

The optimized protocol incorporated all the stepwise improvements listed above (i.e. improved number and duration of extractions, inclusion of a solid phase extraction clean up step, ratio of solvent to soil, and minimum amount of soil for analysis), then compared with the original protocol. The optimized protocol yielded ~30% more ginsenosides however, the increased yield from the original protocol was not a statistically significant amount higher than the old protocol (Figure 3.2) (n = 3, p = 0.06). Future experiments could expand the sample size to determine a better estimate of protocol improvement, and increase the statistical power. Nevertheless, with the improvements to bulk ginsenoside

yield through this optimized protocol, the protocol was next applied to field samples to assess persistence and accumulation of ginsenosides in ginseng gardens.

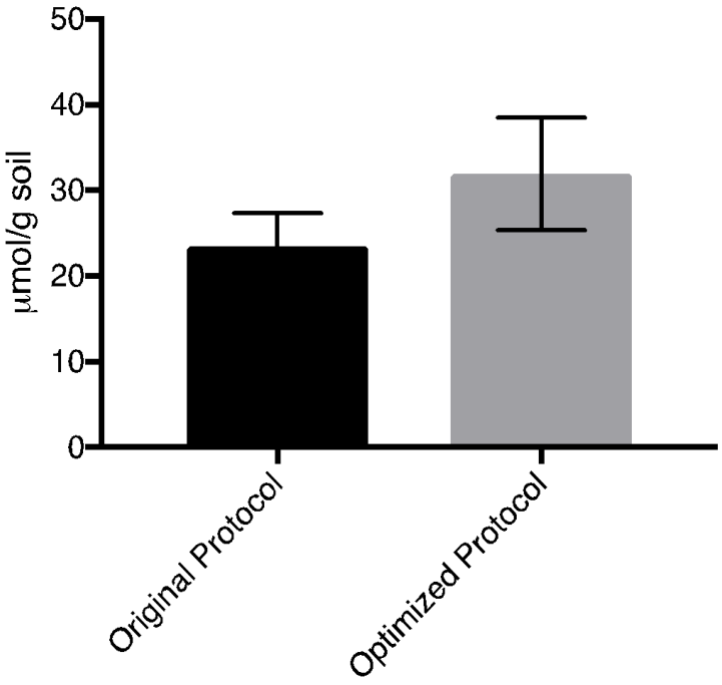

Figure 3.2. Comparison between the original and optimized (mean ± SD) soil extraction protocols of total ginsenosides recovered (µmol/g soil) (n = 3 for each extraction, p = 0.06).

3.2 Field sample analysis

3.2.1 Accumulation of ginsenosides in field samples

The optimized protocol was applied to field samples to quantify and determine the accumulation of ginsenosides in three newly planted commercial ginseng gardens. During the first growing season (2019), only trace amounts of ginsenosides were detected from ginseng garden soils from any of the three sites (Figure 3.3, 3.4, 3.5). In the second growing season (2020), ginsenosides were detected in increasing amounts over the growing season at all three sites (Figure 3.3, 3.4, 3.5). To recall, bulk soil was sampled from 5 different areas within each of the gardens, to obtain an estimate of ginsenoside composition and quantitation. During sampling, destruction of plants was generally avoided, meaning samples were collected at various distances from source roots.

Consequently, for all three garden sites, the amount of the ginsenosides in the soil samples were highly variable.

In site 1, the accumulation of ginsenosides was statistically significant during the second growing season, specifically in Summer B 2019 ($p = 0.0037$) , Fall 2019 ($p = 0.0187$), Spring 2020 ($p = 0.0129$), Summer A 2020 ($p = 0.0028$), Summer B 2020 ($p < 0.001$), Fall 2020 ($p < 0.001$), relative to the control (Figure 3.3, Supplementary table 2). A similar pattern was found at site 2, such that ginsenoside accumulation was statistically significant in the second growing season, specifically Fall 2019 ($p = 0.0224$), Summer B 2020 ($p = 0.0250$) and Fall 2020 ($p < 0.001$) compared to the control (Figure 3.4). Finally, site 3 samples revealed similar statistical significance between control and garden ginsenosides at the Summer A 2020 ($p = 0.0302$), Summer B 2020 ($p = 0.0388$), and Fall 2020 ($p = 0.0029$) collection time (Figure 3.5). For all three sites, the Fall 2020 collection displayed the greatest statistical significance in the amount of ginsenosides in the soil compared to control relative to every other collection time.

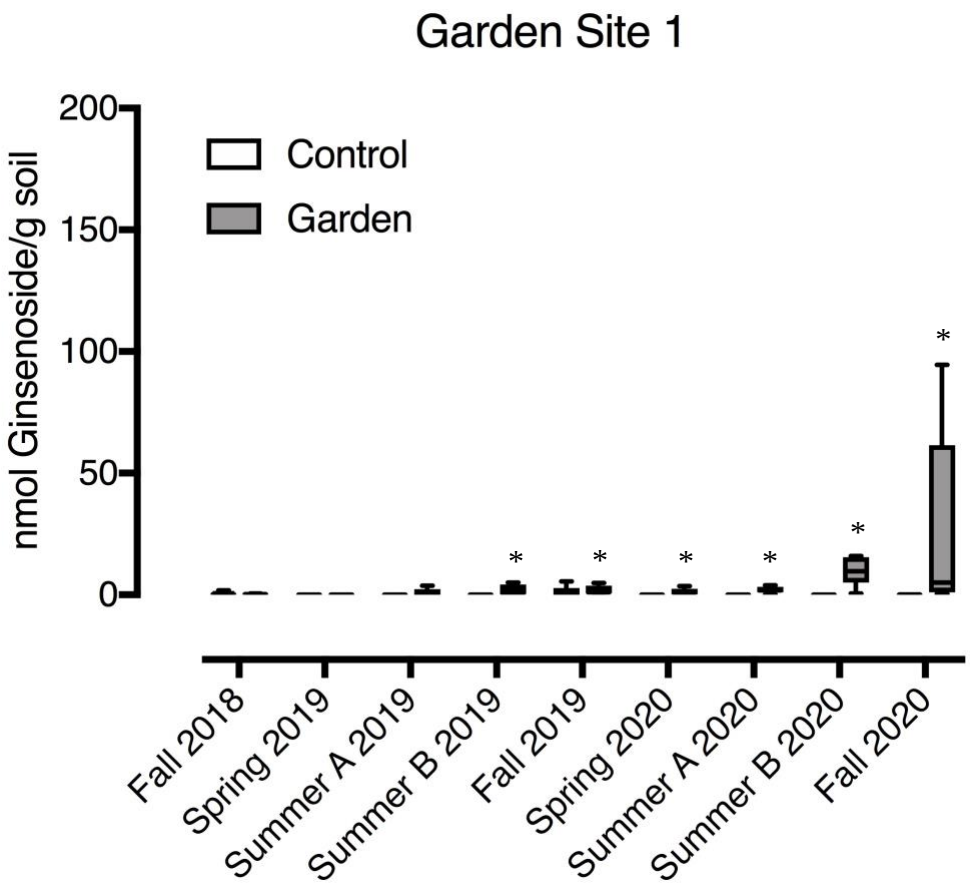

Figure 3.3. Accumulation of ginsenosides in ginseng garden soils in site 1. Total ginsenosides (nmol/g soil) were extracted and analysed from five replicate areas within the garden at each time point. Control samples (n = 5) were collected from the edge of the gardens. Boxes indicate the 25-50 and 50-75 percentiles, while error bars indicate minimum and maximum values. The mean of each sample is represented by the horizontal bar in each box. The asterisk indicates a significant difference (determined by using Tukey's HSD test) in the amount of ginsenosides that were recovered in soil of the ginseng garden (Garden) compared to soil collected on the perimeter of the garden (Control) (GLM: n = 5, df = 8).

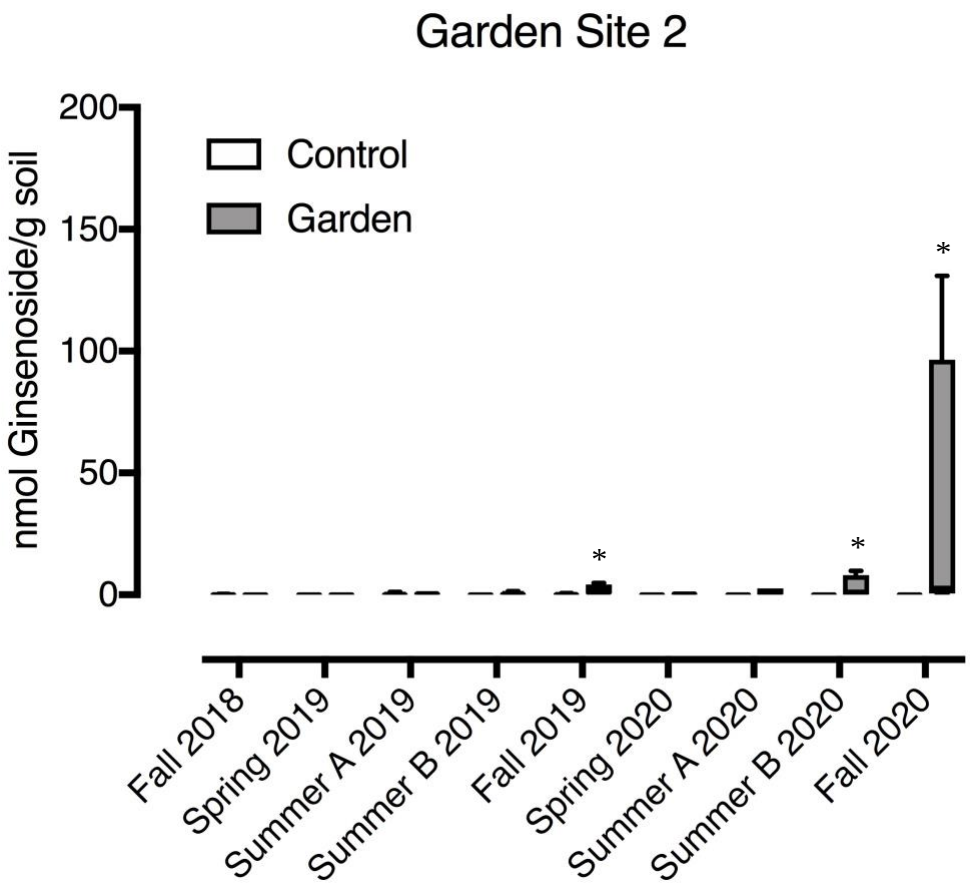

Figure 3.4. Accumulation of ginsenosides in ginseng garden soils in site 2. Total ginsenosides (nmol/g soil) were extracted and analysed from five replicate areas within the garden at each time point. Control samples (n = 5) were collected from the edge of the gardens. Boxes indicate the 25-50 and 50-75 percentiles, while error bars indicate minimum and maximum values. The mean of each sample is represented by the horizontal bar in each box. The asterisk indicates a significant difference (determined by using Tukey's HSD test) in the amount of ginsenosides that were recovered in soil of the ginseng garden (Garden) compared to soil collected on the perimeter of the garden (Control) (GLM: n = 5, df = 8)**.**

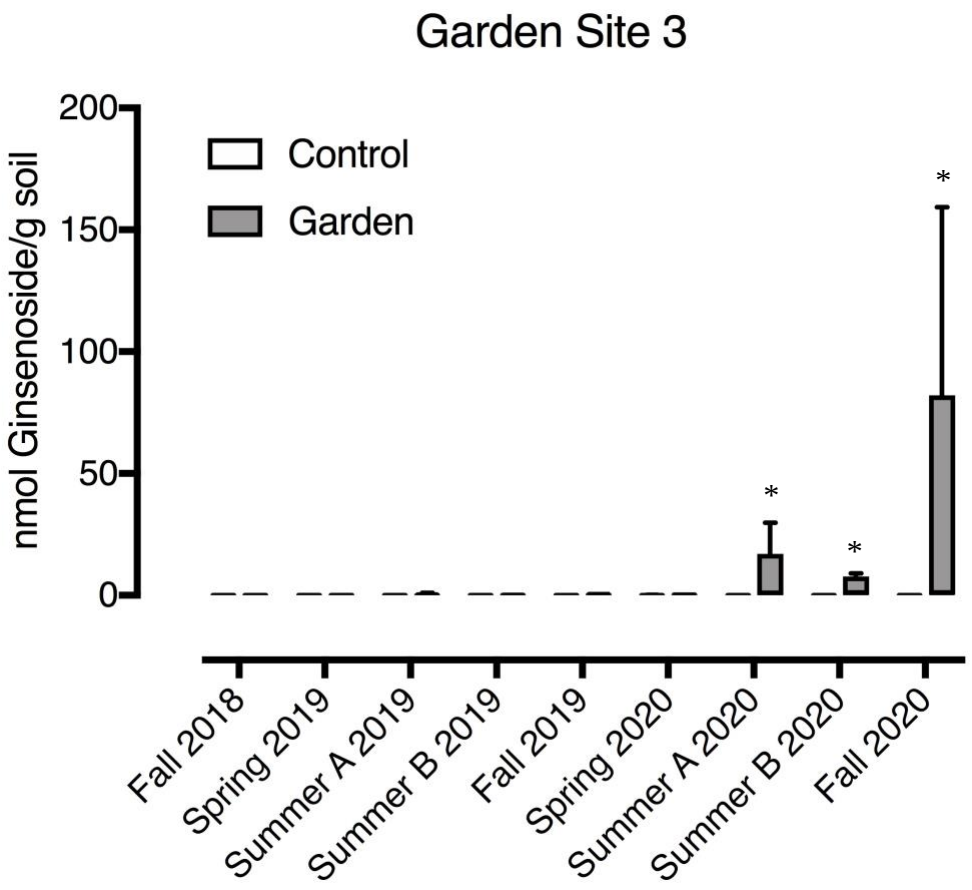

Figure 3.5. Accumulation of ginsenosides in ginseng garden soils in site 3. Total ginsenosides (nmol/g soil) were extracted and analysed from five replicate areas within the garden at each time point. Control samples (n = 5) were collected from the edge of the gardens. Boxes indicate the 25-50 and 50-75 percentiles, while error bars indicate minimum and maximum values. The mean of each sample is represented by the horizontal bar in each box. The asterisk indicates a significant difference (determined by using Tukey's HSD test) in the amount of ginsenosides that were recovered in soil of the ginseng garden (Garden) compared to soil collected on the perimeter of the garden (Control) (GLM: n = 5, df = 8).

Throughout the course of ginseng plant growth and development, ginsenosides are being produced (Court et al., 1996). More specifically, from the moment of germination to a fully mature plant, ginseng plants produce ginsenoside natural products (Court et al., 1996). As the plant ages, the amount of ginsenosides in the plant accumulates, and some

of the ginsenosides produced leach into the surrounding soil (Nicol et al., 2003; Luo et al., 2020). Court et al., (1996) studied how the concentration of ginsenosides increased with root age (in commercial ginseng crops). They found that ginsenoside concentration was highest in plants that were four years old. Moreover, American ginseng crops grown in Ontario (comparable climate region to my research), had yearly increases in root weight and ginsenoside content. In my project, sample collection occurred from the time of germination, until the plants were two years old. Ginsenoside accumulation in commercial crops are known to be the highest at four years old, and with the value of these crops derived from the natural products they produce, harvesting at an older age yields more profit. For the duration of this project, the limited detection of ginsenosides in the field soil could be aligned with the physiological development of the plant, such that in the first two growing seasons, ginsenoside quantities in the plant were low and therefore the amounts leaching into the soil were low as well. This is exemplified by the examination of root ginsenoside content in the roots of plants that were harvested at two years of age. The percent dry weight of ginsenoside content in these two year old roots was 2-3 %, which is half the expected amount of mature ginseng roots. Evidently, at the end of the second growing season, significant amounts of ginsenosides were present in the ginseng gardens relative to the control soil. This corresponds to the accumulation of the compounds in the plants at their oldest age, at the end of the sample collection for this project. As the plants continue to mature and produce these compounds, it is expected that the accumulation of these compounds in the soil will increase as well. My project has analyzed field soils that are two years of age however, determination of accumulation trends will occur as sampling continues in the third and fourth growing season.

Soil composition may also play a factor in the low detection of the compounds. The commercial ginseng gardens were determined to be primarily Loamy-Sand, comprising ~ 75-85% sand. One factor to consider on recovery improvement and ginsenoside detection is the binding capacity of the ginsenosides to this soil. There are various extraneous factors that can contribute to this. In regard to environmental factors, variables like soil pH, soil type, texture, compaction, seasonality, clay content and organic matter may influence the affinity of a compound to soil. There are also chemical factors that need to be considered such as the polarity, interactions with water, charge interactions that can

alter affinity as well (Carter, 2004). Biological factors like microbiome composition, can have an impact on the availability of compounds present in soil while also altering the composition of these compounds in the soil environment (Nicol et al., 2003; Jiang et al., 2019). A combination of these factors can create a complex, yet dynamic soil environment. Ideally, equilibrium would exist and there would be a balance between soil health, microbiome diversity, and plant coexistence however, in the case of ginseng gardens with replant disease, this equilibrium doesn't always exist (Dong et al., 2018). Numerous elements create an environment that favours replant disease conditions in these ginseng gardens. For this project, as the growing seasons progress and microbiome data and ginsenoside analyses continue, a more informative picture will be painted on the relationship among these factors.

To further support the delay in accumulation in soil in this project, a study conducted by Qu et al., (2009), compared different extraction methods to quantify ginsenosides in the roots, leaves, stem, rhizome and root hairs of American ginseng. In particular, they extracted ginsenosides from the roots of one, two, three, four, and five year old roots and found that the total ginsenoside content in these roots (mg/g tissue) continually increased as the plant aged from one to five years of age (~27, 30, 35, 40 and 49 mg/g, respectively). This is important to note as the changes in ginsenoside content in the roots during years one and two were very minimal, which aligns with the age of the roots at the last sample collection for this study (Fall 2020). It is expected that greater differentiation in ginsenoside content in the soil will increase and correspond with the increase in compound production in the roots as the plant ages.

It is known that ginsenosides accumulate in ginseng soil (Yang et al., 2015). However, a deeper understanding on the quantification of these compounds and their role in ginseng replant disease over a time is limited. The relationship between ginsenosides and replant disease was exemplified by the work conducted by Yang et al., (2015), who were able establish that ginsenosides from root exudates, extracts and soil extracts from consecutively cultivated soil had autotoxic effects against the growth and emergence of Sanqi (Chinese) ginseng. They sampled bulk soil from one, two and three years of continuously cultivated fields, and reported total ginsenoside content to be 2.04, 4.16, and

5.87 µg/g soil, respectively. This sampling pattern is comparable to my project where these researchers collected soil from one, two and three year old gardens and conducted ginsenoside analysis. In the fields sampled in my project, total ginsenoside yield in one year old fields ranged from 1.71, 1.86, 0.38 µg/g soil across sites one, two and three (Supplementary table 2, 3, 4). In year two, the total ginsenoside yield in these fields ranged from 24.4, 35.9, 31.1 µg/g soil across sites one, two and three (Supplementary table 2, 3, 4). There are few studies to date that have conducted time course ginsenoside analysis in soils of ginseng gardens. Overall, Yang et al., (2015) report an increase in ginsenoside content in soil over time, and that pattern is emergingly evident in my field data as well.

Autotoxicity plays significant roles in the regulating of community and population densities both in natural and agriculture settings (Batish et al., 2001). In a natural setting, American ginseng produces ginsenosides as a protection mechanism. The driving factor for this may be to control seedling competition, which could ensure the longevity of established plants. This is supported by the fact that ginseng plants are found few and far between in natural settings (Charron and Gagnon, 1991). The aforementioned established plant mechanisms can create issues when the ginseng plants are grown at high density (i.e. in agriculture). When coupled with the influence ginsenosides have on microorganism growth and pathogenicity, it becomes clearer how ginsenosides may influence ginseng production. In agricultural settings, autotoxicity often results in reduction in crop yields, and difficulty establishing or re-establishing plants in soils containing autotoxic compounds. Autotoxicity in American ginseng has been recently confirmed, (He et al., 2009; Yang et al., 2015; Yang et al., 2018). Specifically, exposure of *Panax notoginseng* (Chinese ginseng) roots to ginsenoside Rg1 at increasing concentrations led to a progression of root cell death (Yang et al., 2018). From a cellular standpoint, root exposure to an Rg1 solution increased accumulation of reactive oxygen species (oxidative stress) on ginseng root cells, which lead to reduced cell membrane integrity, damaged root cells and inhibited root growth (Yang et al., 2018). Similarly, Zhang et al., (2011), reported that exposure of American ginseng seedlings to a solution of ginsenosides, a protopanaxadiol mixture and an Rb1 mixture, resulted in inhibitory effects at high concentrations. This was inferred by the reduced function of superoxide

dismutase and peroxidase activities due to exposure to higher concentration of these mixes. The downstream effect of reduced cellular function is the accumulation of reactive oxygen species, and lipid peroxidation of the cell membranes in the roots (Zhang et al., 2011). A more comprehensive analysis of a range of ginsenosides via these mechanisms would provide greater evidence for the effect of ginsenosides on inhibiting root growth. However, to understand the roles ginsenosides play in commercial ginseng gardens specifically, further sampling and analysis must occur in the future growing seasons.

The accumulation of non-ginsenoside compounds may also play a role in contributing to replant disease. Research from Dong et al., (2018), highlighted that ginseng replant disease is a multi-factor, complex system. It is well established that shifts in microbial community composition and diversity can disrupt ecosystem function, equilibrium and overall soil health. In soil environments, shifts in community composition and diversity have the capabilities to alter soil productivity, which can lead to crop/plant death. Dong et al., (2018) describe that environmental and chemical factors such as decline in soil pH, and an accumulation of compounds other than ginsenosides such as cinnamic acid, benzoic acid, and diisobutyl phthalate, which are known to be toxic, and can increase in abundance with continuous ginseng cropping. Biological factors like changes in bacterial diversity (especially a decline) were also noted in continuously cropped ginseng soil. A decline in bacterial taxa that have toxin degrading abilities contributes to the accumulation of non-ginsenoside toxic compounds. As sampling of the gardens monitored in my study progresses in the third and fourth growing season, it may be useful to do a non-targeted compound analysis on extracts from the soils collected to determine whether there are other compounds of interest that may affect soil microbial diversity, and which could cause a shift to a replant disease state. It may be useful to develop a wholistic diagnostic tool that targets different characteristics of soil health such as biological, environmental and chemical factors. If developed thoroughly and comprehensively, this tool could be an informative bioindicator of soil health in ginseng gardens.

The presence of plant derived compounds in soils can drive changes in microbial communities. Jiang et al., (2019) established changes in soil microbial community

composition in fields under continuous ginseng cultivation for four years. By analyzing rhizosphere soil on healthy and diseased roots of American ginseng, they were able to differentiate between the microbiomes of each. Like Dong et al., (2018), Jiang et al., (2019) also found that soil pH was a defining factor in microbial diversity. Soil pH changes in ginseng gardens over subsequent cultivation years were also reported by Tan et al., (2017). Tan et al., (2017) report that initially their fields were neutral to slightly acidic (~pH 5-6), but as continued Chinese ginseng crop rotation occurred, soil became more alkaline (~pH 6-7). Going forward, monitoring soil pH changes in the gardens used in my study may shed light on the potential corresponding microbiome changes as sampling continues in the third and fourth year of this project.

3.2.2 Root profile

Root ginsenoside profiling was conducted to compare the composition of ginsenosides in these roots and determine how well they matched the composition of ginsenosides found in the garden soils. For each site at the last time collection (Fall 2020), roots that were two years of age were collected from the sites and subject to an extraction protocol to determine ginsenosides composition and profile. This information was informative as it provided an idea of the ginsenoside input into the garden soil as well as whether the ginsenosides being produced by the plants were of similar make up and composition to that found in the field soils. The ginsenoside content in American ginseng roots from the gardens studied in my project compares well with other studies, such as Court et al., (1996), who reported the yield of ginsenosides on a total dry weight basis for one, two, three, and four year old roots to be ~3, 4.5, 4.7 and 7.8% respectively, in one set of roots, and ~3, 6.1, 6.8 and 7.5% of total dry weight in another set. Assinewe et al., (2003), determined that total ginsenoside content in four year old roots accounted for ~5.78% dry weight in wild ginseng and ~4.85% in cultivated ginseng. Li et al., (1996) reported 3% ginsenoside content in their roots which were four years of age. In my project, ginsenosides accounted for 2-3% of dry weight in two year old roots, which aligns with what other studies have reported.

Of interest was the proportion of the protopanaxadiols (PPD) and protopanaxatriols (PPT) in the roots compared to the proportion in the field soils. In site 1, the ginsenosides

in the roots had a 2:1 ratio of PPD to PPT (Figure 3.6). For the majority of the time collections, there were significant differences between the proportions of the triols in the root extracts compared to the triols in the soil extracts for Spring 2019 ($p = 0.0001$), Summer A 2019 ($p = 0.013$), Summer B 2019 ($p = 0.0239$), Fall 2019 ($p = 0.0018$) and Fall 2020 ($p = 0.0104$). In the garden soils, however, the ginsenoside pattern was opposite, as there was a 1:2 ratio of PPD to PPT. In site 2, the proportions of triols in the root extracts were significantly different than the proportions of triols in the soil extracts for all time collections, Fall 2018 ($p<0.0001$), Spring 2019 ($p<0.0001$), Summer A 2019 ($p<0.0001$), Summer B 2019 ($p<0.0001$), Fall 2019 ($p = 0.0001$), Spring 2020 ($p<0.0001$), Summer A 2020 ($p = 0.0001$), Summer B ($p = 0.0002$), and Fall 2020 ($p<0.0001$). In site 3, the proportions of triols in the root extracts were significantly different than the proportions in all but the Fall 2019 time collection, Fall 2018 ($p = 0.0001$), Spring 2019 ($p<0.0001$), Summer A 2019 ($p = 0.0004$), Summer B 2019 ($p = 0.0003$), Spring 2020 ($p = 0.013$), Summer A 2020 ($p = 0.0072$), Summer B ($p = 0.0101$), and Fall 2020 ($p = 0.0048$). Overall the 2:1 ratio of PPD to PPT trend was consistent for sites 2 and 3 (Figure 3.7 and 3.8). One possible explanation for the greater proportion of PPT in these field soils are that these compounds are more polar than PPD. This results in greater solubility of the PPT compared to the PPD, which may lead to lower retention to soil particles, and consequently a higher percent recovery. This trend is consistent with data from the optimization experiments, where higher proportions of PPT were recovered from soil in spiked trials compared to PPD.

From a more microbiological perspective, Luo et al., (2020), examined the relationship between different components of root exudates and their impact on the function and composition of various microorganisms. The interaction between ginsenosides and soil microorganisms ultimately acts to shape the soil microbiome. It is well known that ginsenosides enter the soil environment via root exudation (Nicol et al., 2003; Yang et al., 2015) however, Luo et al., (2020) aimed to quantify and identify ginsenosides from the roots of *Panax notoginseng*, and understand the role these compounds have on the soil microbiota. They confirmed that a mixture of ginsenosides (Rg1, Rb1 and Rd, at biologically relevant concentrations found in roots and exogenous root exudates, drove a change in the soil microbiome. More specifically, the mixture of Rg1/Rb1/Rd could

promote the growth of fungal and bacteria isolates. The likely reason for this was the utilization of the ginsenosides as an available carbon resource. The addition of root exudates and the Rg1/Rb1/Rd mixture led to an increase in the richness and diversity of fungi in the soil, and that pattern followed suit for bacteria where Shannon and Simpson indexes also increased post treatment (Luo et al., 2020). Luo et al., (2020) describe the role ginsenosides play in altering the soil microbiome, and how it could shift it to a state that is more characteristic of replant disease conditions. The utilization of ginsenosides as a carbon resource may be a driving factor in altering microbial communities. This could also be a reason for the preferential utilization of PPD by some soil microorganisms (Nicol, et al, 2002, 2003), and thus the lack of them in the field soils, relative to root proportions based on the data from this experiment. The interaction between plant natural products and the uptake or utilization of such by soil microorganisms facilitated by root exudation is a complex and dynamic process. There is continuing mounting evidence to support the role ginsenosides play in altering the soil microbiome, shifting the environment to one that is more characteristics of replant disease.

Li et al., (2020) investigated the impact of Rg1, Rb1 and Rh ginsenosides from Sanqi (Chinese ginseng), on the growth, composition and diversity of soil fungal community. Exposure to these ginsenosides individually and as a mix were shown to alter the fungal microbiome. More specifically, Li et al., (2020) determined that Rg1, Rb1 and Rh enriched taxa pathogenic to ginseng such as *Alternaria, Cylindrocarpon,* and *Fusarium* while decreasing beneficial taxa such as *Mucor, Acremonium* and *Ochroconis* (Li et al., 2020). Structurally, Rb1 and Rh are classified as PPD. The research of Li et al., (2020) support that PPD exposure can alter fungal diversity and influence pathogenic fungi. More specifically, they report that an autotoxic ginsenoside mixture has a synergistic effect on pathogen abundance, they inhibit beneficial taxa, promote fungal abundance and microbial activity (Li et al., 2020). Field data from this study show that there are greater proportions of PPT in the soil, relative to the PPD. Again, this could likely be due to the preferential metabolism of the PPD, which leads to the relative accumulation of more PPT in the soil. The presence and accumulation of PPD and their ability to disrupt the equilibrium of soil fungal communities (Li et al., 2020), still presents an issue with efforts to remediate ginseng replant disease.

To further understand the higher apparent concentrations of PPT in the garden soils, Farh et al., (2017) found that a well-known and established destructive pathogenic fungus to ginseng, *I. mors-panacis*, are sensitive to PPT type ginsenosides, and that the latter may also have an antifungal effect. It has been shown that some pathogenic fungi preferentially metabolize PPD (Ivanov et al., 2016), which may help explain the accumulation of PPT in the garden soil. A detailed microbiome analysis of the gardens surveyed in this project could confirm whether the pathogenic fungi are in greater abundance in garden soils, relative to control soils. Coupling the microbiome identification and classification, with existing research on the preferential metabolic utilization these pathogens have of ginsenosides, can further solidify the role ginsenosides play in ginseng replant disease.

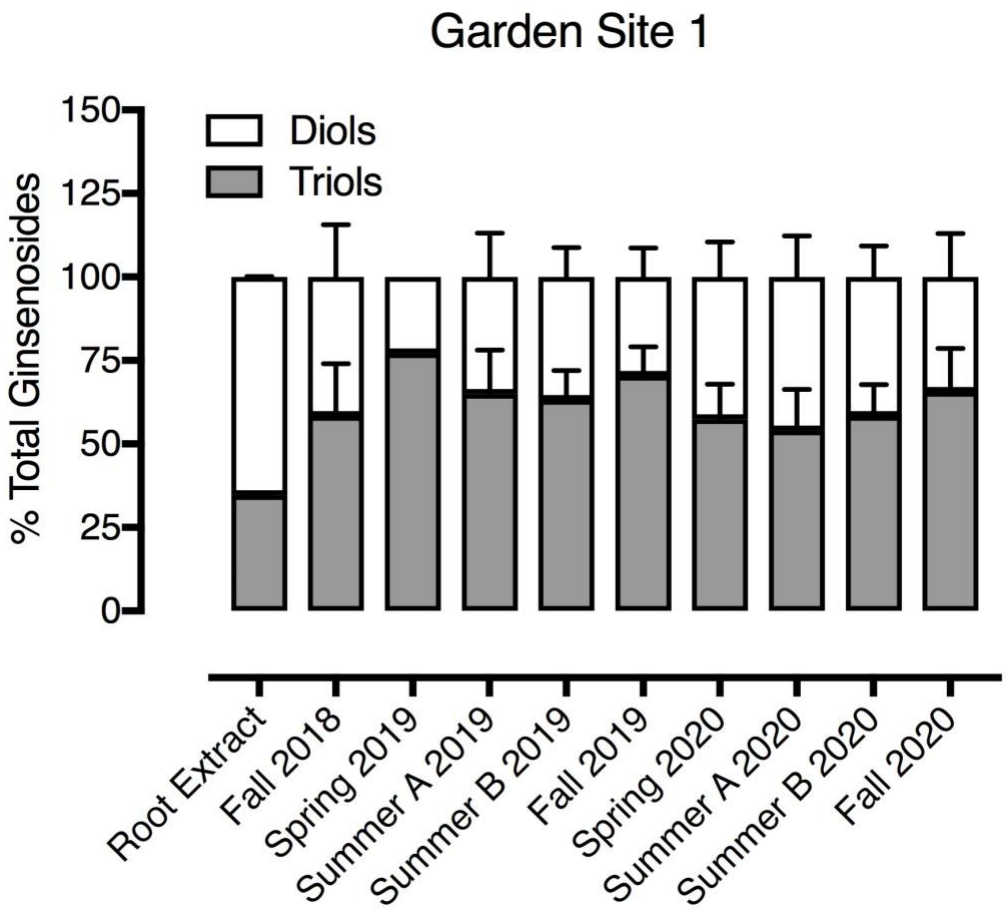

Figure 3.6. Proportion of protopanaxadiol and protopanaxatriol ginsenosides in ginseng roots and garden soils from site 1. Root extract profiles were from two year old ginseng roots obtained from site 1.

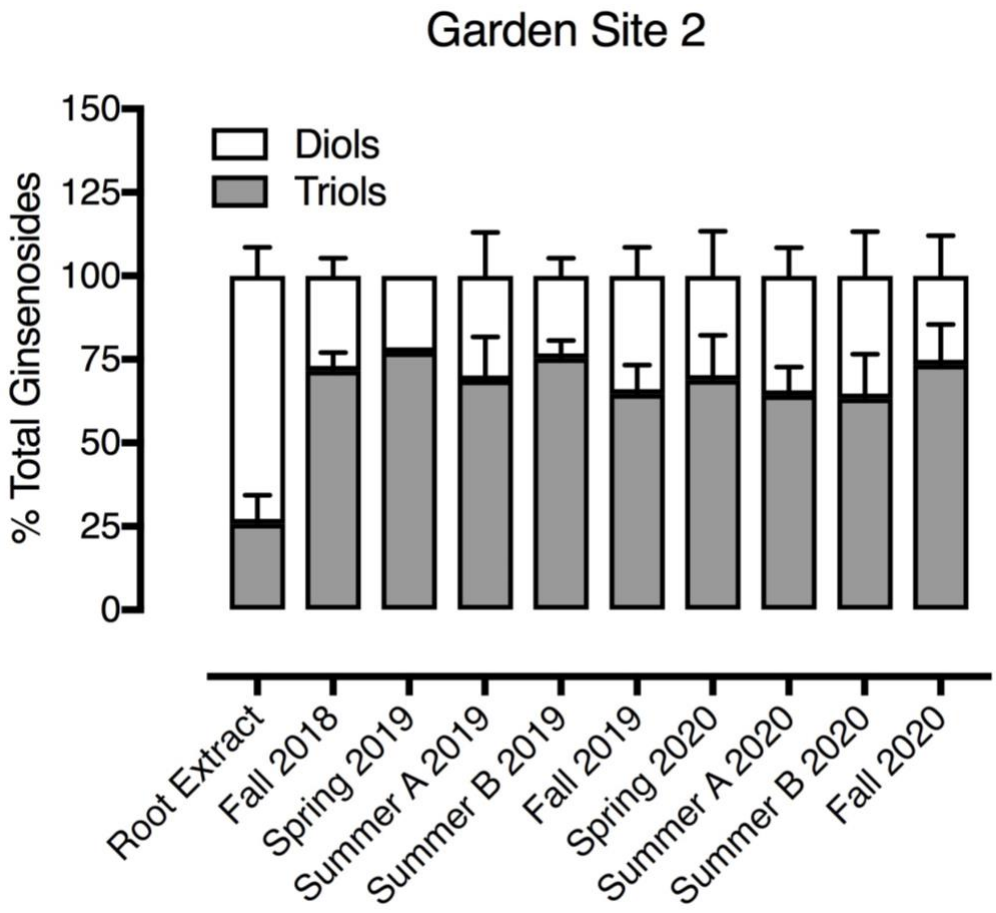

Figure 3.7. Proportion of protopanaxadiol and protopanaxatriol ginsenosides in ginseng roots and garden soils from site 2. Root extract profiles were from two year old ginseng roots obtained from site 2.

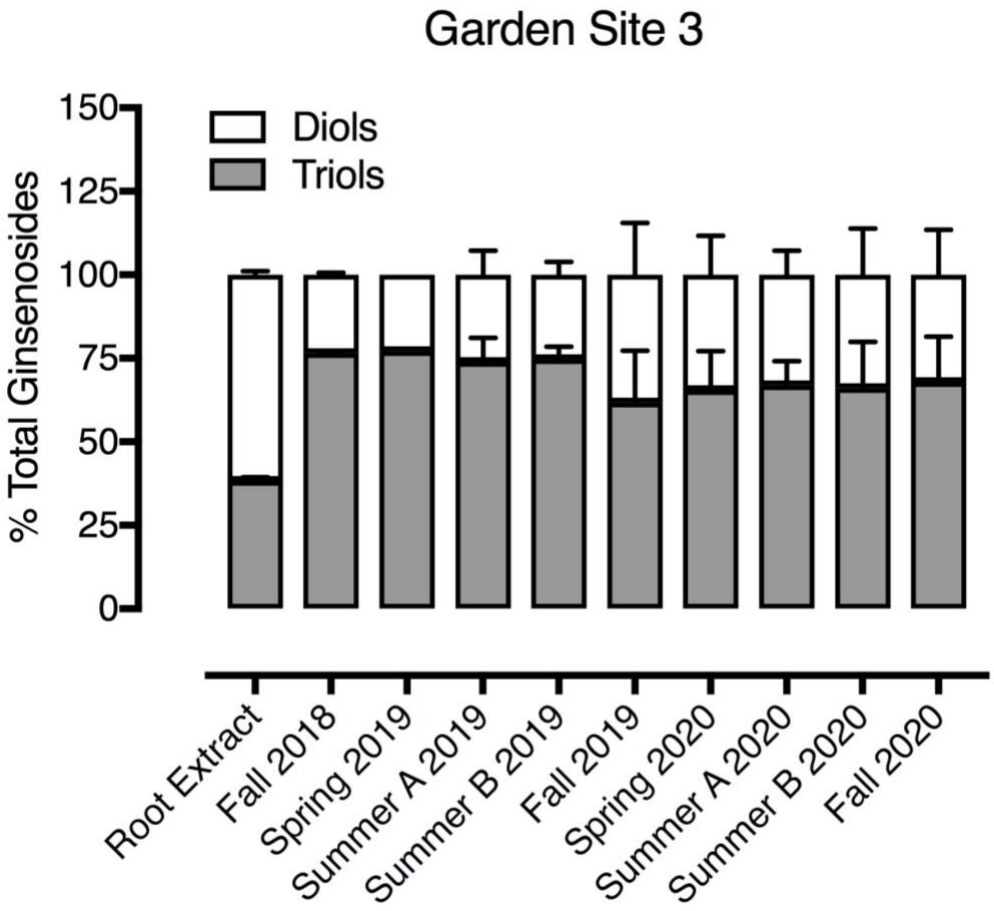

Figure 3.8. Proportion of protopanaxadiol and protopanaxatriol ginsenosides in ginseng roots and garden soils from site 3. Root extract profiles were from two year old ginseng roots obtained from site 3.

3.2.3 Persistence of ginsenosides in harvested ginseng gardens

To evaluate the ability of ginsenosides to persist in soil over time, bulk soil samples were obtained from two research stations (non-commercial ginseng gardens) in Harrow and Vineland Ontario. Both these gardens were harvested in August 2018, and the fields remained unused for three years afterwards. To determine whether ginsenosides persisted in these soils, bulk soils were taken from these fields in October 2018 (approx. one month post harvest), June 2019 and July 2020. In the Harrow ginseng garden, there were no significant factors or interactions, and overall no significant difference between ginsenosides found in the gardens and the control soil found on the perimeter of the field ($p = 0.0526$) (Figure 3.9). In the Vineland ginseng garden, the same pattern remained

such that there was no significant factors or interactions, and no significant difference between the ginsenosides found in the garden and in the control soil ($p = 0.0859$) (Figure 3.10). For both sites, while the quantities were low, there were trace amounts of ginsenosides found in each field. There are many factors that can lead to compounds in soil accumulating and further, persisting. Some of these include compound stability in the matrix, uptake and utilization by microorganisms, natural degradation, and other biological, chemical and environmental factors (Ney, 1995; Ariño et al., 2008). These various factors, independently or combined, could be the root cause for the lack of persistence of ginsenosides in these specific gardens. What is interesting to note is the limited or trace amounts of ginsenosides detected only 2 months post-harvest of three year old roots. Due to the vast possibilities of factors that caused a lack of persistence of ginsenosides in these soils, all that can be reported is that compounds were detected in traceable amounts in these fields.

A logical cause for compound persistence in soil is the presence of plant tissue such as roots, fine root hairs, leaves, stem etc. post-harvest. He at al., (2009), performed soil extractions from soil taken from two fields in China. These fields were former commercial gardens in which American ginseng, had been growing for four years. In their sampling protocol, it was noted that the post-harvest soil had large amounts of fibrous root tissue that remained. They suggested that the autotoxic compounds they found in the soil could partly be derived from these residual root or plant degradation and that over time the compounds may accumulate and contribute to facilitating the autotoxic effects which could hinder growth of American ginseng seedlings in that soil. For my project, the effect of continued accumulation of ginsenosides and residual plant material could be determined or established once the plants continue to mature and approach four years of age. Monitoring of post-harvest soil and observing plant tissue residue may provide greater supporting evidence on the persistence of these compounds over time.

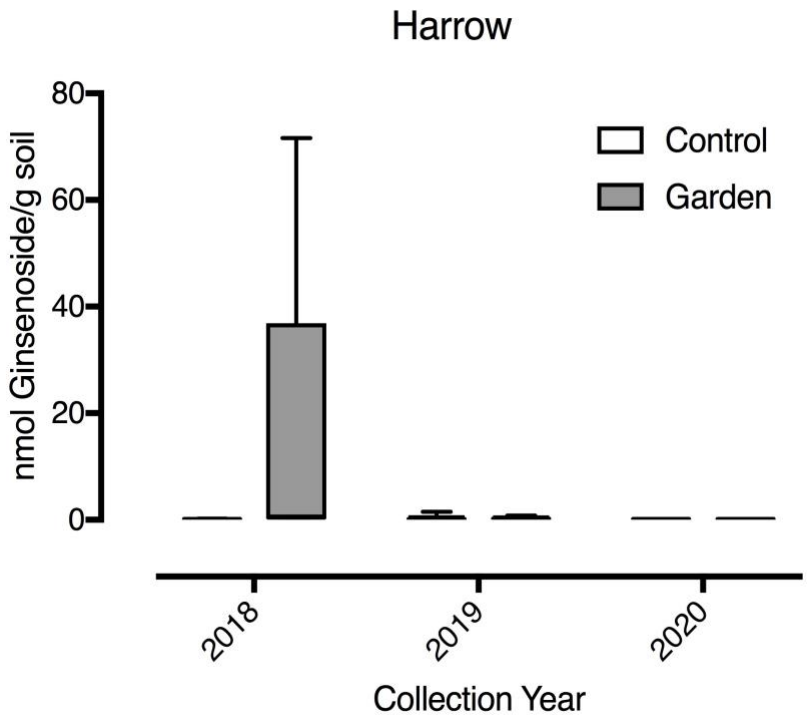

Figure 3.9. Persistence of ginsenosides in ginseng garden soils from Harrow, Ontario. Total ginsenosides (nmol/g soil) were determined for bulk soils collected in the year of harvest and two subsequent years. The data display the average of five replicates (n = 1) of soils from the ginseng garden (Garden) compared to soil collected on the perimeter of the garden (Control) **(**GLM: n = 1, df = 1).

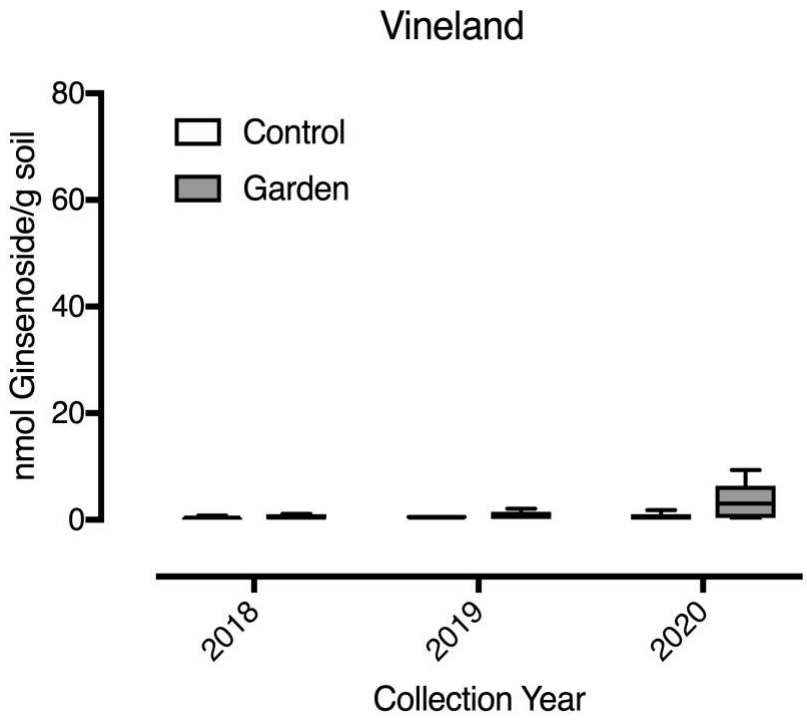

Figure 3.10. Persistence of ginsenosides in ginseng garden soils from Vineland, Ontario. Total ginsenosides (nmol/g soil) were determined for bulk soils collected in the year of harvest and two subsequent years. The data display the average of five replicates (n = 1) of soils from the ginseng garden (Garden) compared to soil collected on the perimeter of the garden (Control) **(**GLM: n = 1, df = 1).

3.3 Future directions and conclusions

The data collected from commercial ginseng garden soils using my protocol optimization still highlights a need to improve inconsistencies with reproducibility. To date, there are still outstanding issues with the ability to completely recover ginsenosides from soil. This leads to downstream issues with accuracy in ginsenoside detection. Despite a 30% increase in overall yield using my optimized protocol, more work needs to be done to isolate and target the variables that hinder a higher percent recovery. These could be exploring soil binding capacities, specific to soils where ginseng is grown, further exploration into breakdown products and transformations of the main ginsenosides found in ginseng roots, as this could influence what is detected in the soil. Furthermore, exploring a more encompassing or representative sampling procedure may help with

gaining a more accurate representation of ginsenoside accumulation in these fields, while also assist with reducing the variability that arises with bulk sampling. The data from my research provide a foundational improvement in the area pertaining to the extraction and detection of ginsenosides from soil. This will be a critical tool in research surrounding ginseng replant disease, particularly in the determination of ginsenoside content in both commercial gardens and future field evaluations.

Using my optimized protocol, ginsenoside accumulation was measured over the first two years of cultivation in newly planted ginseng gardens. As ginseng is a slow growing plant, the ability for the plant to produce and release ginsenosides into these soils to subsequently accumulate is also slow. Future data from the third and fourth growing seasons will yield a better picture of the accumulation of ginsenosides in ginseng garden soil, as the plants become more mature and produce more ginsenosides. On the other hand, based on my analysis, the persistence of ginsenosides (as measured in soils from recently harvested garden sites) does not appear to follow a specific pattern. Moreover, ginsenosides don't seem to remain in the soil for long periods of time; however, this could be related to the fact that at the time of harvest, there were only trace amounts of ginsenosides present in these gardens.

Overall, the field data collected for my project depicts high variability within in a site, and that this characteristic is consistent at each field site. One hypothesis is that the high level of variability in ginsenoside content in replicate bulk soil samples from the same garden reflects the non-uniform distribution of ginsenosides within these garden soils. Nevertheless, my work to improve ginsenoside extraction from soil provided some optimization and improved efficiencies. My project was based on the premise that ginsenosides play a role in ginseng replant disease. The multitude of research published supports the idea that ginsenosides play a functional role in the progression of a ginseng garden to a replant disease state. The significance of my project is that it will allow researchers to extract ginsenosides with better efficiency from ginseng garden soils, in new or previously used fields, which can in part, inform on the potential of a field to move to a replant diseased state, or one that is suitable for growing ginseng.

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Appendix

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SOIL TEST REPORT

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Sample Number	Lab Number	Organic Matter	Phosphorus - P ppm Bicarb	Phosphorus - P ppm Bray-P1	Potassium K ppm	Magnesium Mg ppm	Calcium Ca ppm	Sodium Na ppm	pH	pH Buffer	CEC meq/100g	Percent Base Saturations % K	% Mg	% Ca	% H	% Na
S1	55568	1.6	134 H	397 H	213 H	169 M	1680 H	16 L	7.2		10.9	5.0	12.9	76.9	4.6	0.6
S2	55569	2.4	85 H	236 H	211 VH	208 H	900 M	15 M	6.8	6.9	8.0	6.7	21.6	56.1	14.7	0.8
S3	55570	1.1	91 H	262 H	84 M	98 M	1340 VH	17 M	7.3		7.8	2.8	10.5	86.0		0.9

Sample Number	Sulfur S ppm	Zinc Zn ppm	Manganese Mn ppm	Iron Fe ppm	Copper Cu ppm	Boron B ppm	Soluble Salts ms/cm	Saturation %P	Aluminum Al ppm	Saturation %Al	Nitrate Nitrogen NO3-N ppm	K/Mg Ratio	ENR	Field ID
S1								37 H	1379	0.2 G		0.39	28	
S2								27 H	1133	0.4 G		0.31	36	
S3								20 H	1310	0.2 G		0.27	23	

OE VL = VERY LOW, L = LOW, M = MEDIUM, H = HIGH, VH = VERY HIGH, G = GOOD, MA = MARGINAL, MT = MODERATE PHYTO-TOXIC, T = PHYTO-TOXIC, ST = SEVERE PHYTO-TOXIC

SOIL FERTILITY GUIDELINES (lbs/ac)

Sample Number	Crop	Yield Goal	Lime Tons/Acre	N	P2O5	K2O	Mg	Ca	S	Zn	Mn	Fe	Cu	B
S1	Ginseng (planting)	lbs	0.0	40	50	50	15	40						
S2	Ginseng (planting)	lbs	0.0	40	50	50	0	300						
S3	Ginseng (planting)	lbs	0.0	40	50	205	25	0						

Supplementary Figure 1. Soil test report (conducted and supplied by A & L laboratories Inc.) for soil collected at 3 commercial ginseng gardens (site 1-3).

Supplementary Table 1. Solvent test data. Test parameters include 100% MeOH, 100% acetonitrile (ACN), and 100% MeOH + 2 mL ACN wash.

Ginsenosides	MeOH wash only			ACN wash only			MeOH wash + 2 mL ACN wash					
	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3
	Peak area			Peak area			Peak area			Peak area		
Rb1	75619	69145	42386	28267	29441	26723	6540	57580	65104	0	0	0
Rb2	41968	38451	22398	15371	14256	14133	34450	31305	34707	0	0	0
Rc	639117	58598	35347	17671	18142	17972	50732	48701	55445	0	0	0
Rd	37179	33474	19248	8753	8597	8112	28401	27584	30654	0	0	0
Gyp. XVII	69261	64333	36433	13369	13235	12411	52896	53064	58174	0	0	0
F2	62253	60784	33692	7255	7137	6656	40210	47731	52817	0	0	0
F11	105454	106096	65048	79941	54863	70064	126473	110267	110011	0	0	0
Rg1	222724	227784	154569	214451	116347	196258	299036	272280	263693	0	0	0
Re	88726	87910	61505	78356	42727	74104	113203	101035	98919	0	0	0

Supplementary Table 2. Site 1 control and garden total ginsenoside quantities (nmol/g). Quantities shown are for each of the five sampling areas for all nine time collections. Total values in last column represent total ginsenoside content in the soil after the first and second growing season. Values are converted into units (µg/g) for literature comparison.

Control	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.3902	1.8182	0.2990	0.2362	0.2277	1.16
Spring 2019	0.1308	0.1543	0.1306	0.1291	0.1299	
Summer A 2019	0.1291	0.1291	0.1295	0.1291	0.1302	
Summer B 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Fall 2019	5.6069	0.1291	0.1291	0.1291	0.1291	
Spring 2020	0.1291	0.1291	0.1291	0.1291	0.1291	0.15
Summer A 2020	0.1349	0.1354	0.1338	0.1352	0.1348	
Summer B 2020	0.1362	0.1384	0.1366	0.1352	0.1367	
Fall 2020	0.1314	0.1291	0.2921	0.1291	0.1291	
Garden	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.2669	0.1645	0.5271	0.1472	0.2967	1.71
Spring 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Summer A 2019	0.7537	3.7801	0.1300	0.3347	0.1381	
Summer B 2019	0.6175	5.0309	0.7908	0.2950	3.3204	
Fall 2019	0.8744	4.8811	1.0494	0.6176	2.5523	
Spring 2020	0.4730	3.5609	0.8142	0.1488	1.5285	24.44
Summer A 2020	0.1359	1.8299	2.6017	1.7921	3.9667	
Summer B 2020	0.5215	15.0778	16.0136	9.7330	9.6648	
Fall 2020	5.1099	0.3372	94.4896	1.7572	28.5340	

Supplementary Table 3. Site 2 control and garden total ginsenoside quantities (nmol/g). Quantities shown are for each of the five sampling areas for all nine time collections. Total values in last column represent total ginsenoside content in the soil after the first and second growing season. Values are converted into units (µg/g) for literature comparison.

CONTROL	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.4621	0.1469	0.1368	0.1391	0.1383	0.22
Spring 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Summer A 2019	0.1291	0.1291	0.1291	1.1336	0.1291	
Summer B 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Fall 2019	0.1291	0.1291	0.1291	0.6604	0.1291	
Spring 2020	0.1291	0.1291	0.1291	0.1291	0.1291	0.12
Summer A 2020	0.1366	0.1353	0.1404	0.1380	0.1475	
Summer B 2020	0.1342	0.1346	0.1347	0.1344	0.1343	
Fall 2020	0.1339	0.1291	0.1407	0.1291	0.1291	
GARDEN	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.1586	0.1326	0.1379	0.1422	0.1291	1.86
Spring 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Summer A 2019	0.8737	0.9013	0.1291	0.4253	0.2296	
Summer B 2019	0.1291	1.5670	0.2586	0.7720	0.1291	
Fall 2019	4.9142	3.4746	0.7459	0.2866	0.8388	
Spring 2020	0.1291	0.6631	0.1291	0.3333	0.6299	35.93
Summer A 2020	2.6191	0.6021	0.4561	2.5860	0.1386	
Summer B 2020	6.2841	9.7836	0.3251	0.1810	0.1657	
Fall 2020	0.8603	0.2801	130.9556	2.8537	61.8427	

Supplementary Table 4. Site 3 control and garden total ginsenoside quantities (nmol/g). Quantities shown are for each of the five sampling areas for all nine time collections. Total values in last column represent total ginsenoside content in the soil after the first and second growing season. Values are converted into units (µg/g) for literature comparison.

CONTROL	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.1321	0.1303	0.1304	0.1320	0.1291	0.12
Spring 2019	0.1321	0.1298	0.1291	0.1296	0.1291	
Summer A 2019	0.1291	0.1291	0.1291	0.1291	0.1301	
Summer B 2019	0.1291	0.1291	0.1291	0.1291	0.1301	
Fall 2019	0.1291	0.1291	0.1300	0.1291	0.1300	
Spring 2020	0.1291	0.1602	0.1291	0.1291	0.1416	0.12
Summer A 2020	0.1367	0.1383	0.1372	0.1376	0.1372	
Summer B 2020	0.1342	0.1346	0.1347	0.1344	0.1343	
Fall 2020	0.1291	0.1291	0.1291	0.1291	0.1291	
GARDEN	Area 1	Area 2	Area 3	Area 4	Area 5	Total Year 1 & 2 (µg/g)
	Sum (nmol/g)					
Fall 2018	0.1317	0.1298	0.1291	0.1308	0.1291	0.38
Spring 2019	0.1291	0.1291	0.1291	0.1291	0.1291	
Summer A 2019	0.1291	0.1291	1.0694	0.1291	0.1291	
Summer B 2019	0.2933	0.1367	0.1291	0.1386	0.1404	
Fall 2019	0.1291	0.1697	0.9523	0.1291	0.7132	
Spring 2020	0.1291	0.2378	0.1935	0.1291	0.4214	31.11
Summer A 2020	0.4882	4.2463	29.8310	0.1367	0.1357	
Summer B 2020	6.2841	9.1125	0.3332	0.1793	0.1852	
Fall 2020	0.2915	0.2592	0.7716	159.3834	4.8087	

Curriculum Vitae

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Work Experience
Teaching Assistant
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Academic Experience
Society of Graduate Students (SOGS) Councillor
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Publications:

Woolfson, K.N., Haggitt, M.L., Zhang, Y., Kachura, A., Bjelica, A., Rey Rincon, M.A., **Kaberi, K.M.** and Bernards, M.A. (2018). Differential induction of polar and non- polar metabolism during wound-induced suberization in potato (*Solanum tuberosum* L.) tubers. *The Plant Journal* **93**(5), 931-942.

Non-Peer reviewed Publications:

Kaberi, K.M., and Bernards, M.A. (2020). Analysis of ginsenosides from ginseng garden soil: Optimization. Ontario Ginseng Research Report, Ontario Ginseng Growers Association, in press.

Kaberi, K.M., and Bernards, M.A. (2021). Analysis of ginsenosides from ginseng garden soil: Field analysis. Ontario Ginseng Research Report, Ontario Ginseng Growers Association, in press.

Presentations:

Kaberi, K.M. (2020). The accumulation and persistence of ginsenosides in ginseng garden soil. Ginseng Replant Working Group Workshop, Simcoe, Ontario (oral).

Academic Meetings:

Ginseng Replant Disease Working Group Meeting. Hosted by Sean Westerveld, Ginseng Specialist for the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA). Attended: 09/2018, 01/2019, 09/2019, 01/2020, 02/2021