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Ginsenoside Variation and Phytochemistry of Ontario-Grown North American Ginseng (*Panax Quinquefolius*): Assessing Land Race Diversity and Biological Activities

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GINSENOSIDE VARIATION AND PHYTOCHEMISTRY OF ONTARIO-GROWN NORTH AMERICAN GINSENG (*PANAX QUINQUEFOLIUS*): ASSESSING LAND RACE DIVERSITY AND BIOLOGICAL ACTIVITIES

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ABSTRACT

North American ginseng (*Panax quinquefolius* L.) is a valuable and widely used medicinal plant and Ontario has become the largest grower of ginseng in North America. Ginseng has been grown in Ontario for over 50 years and has reached the land race stage. The phytochemistry of Ontario ginseng land races was characterized using high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD), evaporative light scattering detector (ELSD), or mass spectrometry (MS) to assess ginsenoside and monosaccharide content. Nuclear magnetic resonance (NMR) was used successfully as a metabolomic tool to distinguish Ontario ginseng land races and ginseng species. Ginsenoside variation was high within and among Ontario ginseng land races and variation in ginsenoside content was correlated positively to the level of inhibition of the drug metabolizing cytochrome P450 enzyme, CYP3A4. Along with the assessment of ginsenosides, LC/MS/MS and LC/ELSD methods were developed to characterize malonyl ginsenosides and monosaccharide components respectively in Ontario ginseng. Malonyl ginsenosides accounted for a significant percentage of total ginsenoside content and glucose was found to be the major monosaccharide component. Although chromatographic ginsenoside analysis did not differentiate land races, ^{1}H NMR was successfully applied to distinguish ginseng species and two of five land races. The results from this study contribute to the validation and characterization of Ontario ginseng and add to the value of this important medicinal crop.

RÉSUMÉ

Le ginseng (*Panax quinquefolius* L.) nord-américain est une plante médicinale à valeur élevée et grandement utilisée et dont l'Ontario est devenu le plus grand producteur en Amérique du Nord. Cette plante est cultivée en Ontario depuis plus de 50 ans et a atteint le stage de lignées. La phytochimie des lignées de ginseng de l'Ontario a été caractérisée dans cette étude par chromatographie liquide à haute performance (*HPLC*) couplée à un détecteur à rayons diodiques (*DAD*), ainsi qu'à un détecteur à dispersion lumineuse évaporatrice (*ELSD*) ou à un spectromètre de masse (*MS*) afin de déterminer les teneurs en ginsénosides et en monosaccharides. La résonance magnétique nucléaire (*NMR*) s'est révélée être un outil métabolomique de choix pour distinguer les lignées de l'Ontario et autres espèces de ginseng. La variation des ginsénosides était élevée dans et entre les lignées de ginseng de l'Ontario et la variation dans les teneurs en ginsénosides a été corrélée positivement avec le degré d'inhibition du composé métabolisant l'enzyme CYP3A4 cytochrome P450. En plus de la détermination des ginsénosides, les méthodologies *LC/MS/MS* et *LC/ELSD* ont été développées afin de caractériser, respectivement, les ginsénosides-malonyl et les composantes monosaccharidiques du ginseng de l'Ontario. Les ginsénosides-malonyl étaient, en pourcentage, les plus significativement élevés par rapport à la teneur totale des ginsénosides, le glucose étant le plus important parmi les monosaccharides. Bien que l'analyse chromatographique des ginsénosides n'a pu différentier les lignées de ginseng, l'analyse par ^{1}H *NMR* a distingué les espèces de ginseng et deux des cinq lignées. Les résultats de cette étude contribuent non seulement à la validation et à la caractérisation du ginseng de l'Ontario mais rendent encore plus compte de sa valeur comme plante médicinale.

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LIST OF ABBREVIATIONS

1YM- 1 year old micropropagated
4YM- 4 year old micropropagated
4YS- 4 year old seed derived
AGE- advanced glycation endproduct
ARA- arabinose
BSA- bovine serum albumin
COSY- correlation spectroscopy
CPMG- Carr-Purcell-Meiboom-Gill
CV- coefficient of variation
CYP- cytochrome
DBF- dibenzylfluorescein
DMSO-d6- deuterated dimethyl sulfoxide
DPPH- 2,2-diphenyl-1-picrylhydrazyl
DSS- 4,4- dimethyl-4-silapentane-1-sulfonic acid
EMS- enhanced mass scan
GAL- galactose
GAL A- galacturonic acid
GLU- glucose
GSH- reduced glutathione
GSSG- glutathione disulfide
HPLC-DAD- high performance liquid chromatography- diode array detector
HPLC-ELSD- high performance liquid chromatography- evaporative light scattering detector
HSQC- heteronuclear single quantum correlation
IC_{50}- inhibitory concentration of fifty percent activity
JRESQF- J-resolved
LC/MS/MS- liquid chromatography/ mass spectrometry/ mass spectrometry
MFC- methocy-4 trifluoromethylcoumarin
MRM- multiple reaction monitoring
NADPH- reduced B-nicotinamide adenine dinucleotide phosphate
NMR- nuclear magnetic resonance
NOESY- nuclear overhauser effect spectroscopy
RHA- rhamnose
T2D- type 2 diabetes mellitus
TCM- traditional Chinese medicine

CHAPTER 1

1.0 INTRODUCTION

1.1 INTRODUCTION TO THESIS

Ginseng is Ontario's most important medicinal crops and Ontario has become the largest grower of ginseng in North America. Ontario ginseng has reached the land race stage, which is a farmer selected variety that has diverged significantly from wild populations, growing separately for several decades. Although Ontario ginseng is worth approximately $100 million annually, it has not been extensively characterized phytochemically. Furthermore, ginseng land races have not been examined and each land race may have unique phytochemical or biological characteristics. Ginsenosides are thought to be the main constituents contributing to the wide-ranging biological activity of ginseng, though polysaccharides and other constituents have also been shown to have important activities. The main focus of this thesis is to improve the characterization of Ontario ginseng, assessing ginsenoside variation as well as analyzing malonyl ginsenoside and monosaccharide components. Phytochemical variation was also assessed in relation to biological activities and methods to rapidly distinguish ginseng species and land races for routine analysis were examined.

1.2 LITERATURE REVIEW

1.2.1 Ginseng use in traditional medicine

Ginseng has been used in Traditional Chinese Medicine (TCM) for centuries to replenish vital energy, or "qi." It is used to treat a variety of ailments including fatigue and

insomnia, and chronic illnesses such as diabetes (Chinese Pharmacopoeia Commission, 2005). Ginseng is a member of the genus *Panax* genus (Araliaceae family). Of the 13 reported ginseng species, North American *Panax quinquefolius* L., and Asian *Panax ginseng* C.A. Meyer, are the most important in cultivation and are widely used natural health products (Bai et al., 1997). In TCM, yin and yang are opposite but complementary forces and prolonged yin deficiency leads to a deficiency in both yin and yang and overall "qi" (Li et al., 2004). Traditionally, American ginseng is associated with "yin", or cooling properties, whereas Asian ginseng is associated with "yang", or warming properties. Aside from their traditional properties, these species differ based on their natural geographical distribution and the composition of their active constituents.

Along with an extensive history of use in TCM, *P. quinquefolius* root has been traditionally used among aboriginal peoples of North America. It has been recorded as a general tonic and is specifically used to treat fever, stomach pain, nose bleed, and shortness of breath, as well as to increase mental powers (Arnason et al., 1981;Foster, 1996). Ginseng is recognized in the European Commission E monographs as an effective treatment for fatigue or declining work capacity at a dose of 1-2 g of root/ day for up to 3 months (Blumenthal, 2003).

1.2.2 Botanical characteristics and life history

American ginseng is a perennial plant which grows from a taproot and stands at 30-50 cm. It contains 3 long-stalked leaves which consist of a petiole and five palmately divided leaflets. Ginseng has a rhizome as well as a root system. The root system consists of main roots, lateral roots, and root hairs (Figure 1. 1) (Christensen et al., 2006)). A typical plant

will contain several taproots along a thin rhizome, which is characterized by annual scars from abscission of the aerial stem (Charron and Gagnon, 1991). The primary root acts as a fleshy storage root and adventitious roots form at nodes along the rhizome. Dry weight gain in ginseng roots is recorded to be approximately 0.2 g in seedling, 2 g in two-year-old roots, and 3.5 g for three-year-old roots over the growing season from mid-June to mid-September (Proctor et al., 2003).

Figure 1. 1. A representative 4 year old Ontario ginseng root (*P. quinquefolius*) consisting of main roots, lateral roots, and root hairs.

American ginseng is a slow growing plant, generally requiring 4 years of growth before harvest. Ginseng is primarily propagated by seed, which requires a stratification

period of 18-22 months under cool temperature in order for germination to occur. The seedling shoot has a trifoliate leaf and the petiole is approximately 20% of its expected height. Two- and three- year old ginseng stems develop from an apical perennating bud on the rhizome after the required cold dormancy period (Proctor et al., 2003). After three pre-reproductive years, white or yellowish-green flowers become present in July and August and white berries appear in clusters where leaves are arranged around the stem (MacKinnon et al., 2009;Charron and Gagnon, 1991;Proctor et al., 2003).

P. quinquefolius has a mixed mating system, whereby flowers are self-compatible but cross-pollination with other plants is also possible (Lewis and Zenger, 1983;Mooney and McGraw, 2007). In one study, the only differences noted between self-pollinated versus cross-pollinated plants were a higher proportion of seeds produced from self-pollinated flowers and smaller leaf area and plant height in 2 year old seedlings of self-pollinated compared to cross-pollinated plants (Mooney and McGraw, 2007). In general, self-pollination is more likely to occur in wild stands which have low population density and high disturbance.

1.2.3 Distribution, status andhHistory of cultivation

In 1716 North American ginseng was recognized as a relative of Asian ginseng by Joseph Lafitau, a Jesuit priest who had been a missionary in China. American ginseng was recognized as distinct from the Asian species in TCM. American ginseng is endemic to the Eastern North America deciduous forest biome. In Canada it grows from southern Quebec to southern Ontario and it is also distributed through much of the Eastern United States as far south as Louisiana (Figure 1. 2) (United States Department of Agriculture, 2009). It can be

found primarily in mixed deciduous forests where *Acer saccharum* (sugar maple) is the dominant over story species and occurs in a wide range of soils varying from high sand to high silt to high clay. *P. quinquefolius* populations are greatly effected by disturbance and most stands occur in old growth primary forest areas with little or no habitat disruption (Anderson et al., 1993).

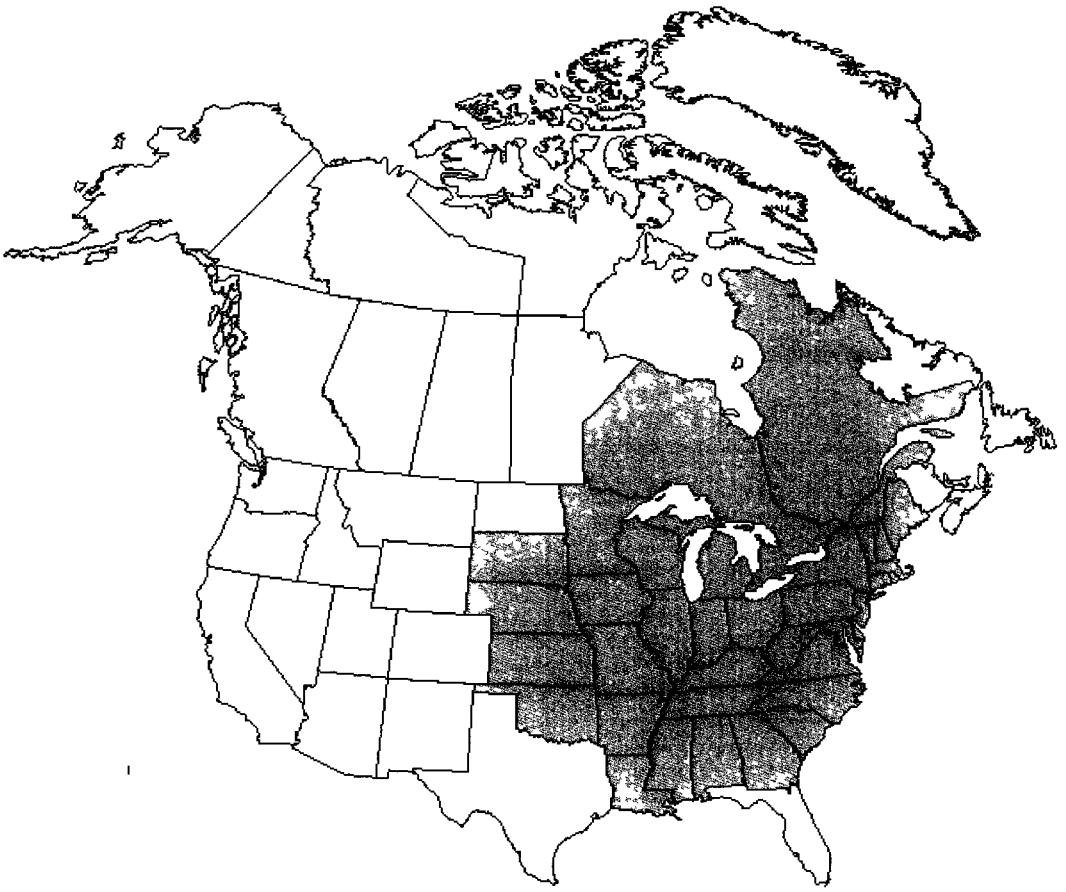

Figure 1. 2. Natural distribution of *Panax quinquefolius* in North America (provinces and states of occurrence highlighted, but distributions may be limited to a small region of the province/state, for example Southern Ontario and Quebec). This figure was modified from the USDA Plants Profile of *Panax quinquefolius* (United States Department of Agriculture, 2009).

When wild ginseng was discovered in North America, great amounts were exported to China and other Asian markets where North American *P. quinquefolius* was recognized as distinct from Asian *P. ginseng*. As the demand grew, wild American ginseng was harvested so extensively in the 18th and 19th centuries that it nearly became extinct (Carlson, 1986). Due to over-harvesting and deforestation of its natural habitat (old growth forest), American ginseng is now considered a threatened species and is listed in Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Carlson, 1986;Schlag and McIntosh, 2006;Burkhart and Jacobson, 2009).

In response to the threatened status of ginseng, cultivation began in order to meet export demands. In Ontario, ginseng cultivation began in the late 1800s with the Hellyer family (Ontario Ginseng Growers Association, 2009). Seeds were originally obtained from wild root and cultivation in Ontario has grown to include close to 400 growers with approximately 6000 acres of land in production and 1.8 million kilograms of root harvested annually. Today Ontario ginseng has reached the land race stage, which is a farmer selected variety that has diverged significantly from wild populations. Cultivated American ginseng is one of North America's most valuable medicinal crops, worth an estimated $100 million annually in farm sales, and is also one of the top selling dietary supplements in the United States (Small and Catling, 2003;Mihalov et al., 2000). Ontario is the largest producer of ginseng in North America, responsible for approximately half of the supply (Roy et al., 2003). Most cultivated ginseng continues to be exported to Asian markets. In the marketplace ginseng sells as raw root, root powder in capsules, extracts, tablets and tinctures. The most valuable commercial product is CV Technologies Cold FX, a clinically tested American ginseng polysaccharide derivative commanding a $48 million dollar market.

1.2.4 Phytochemical constituents of ginseng

1.2.4.1 Ginsenosides

Ginseng contains many types of secondary metabolites including ginsenosides, polysaccharides, and polyacetylenes, though most of the biological activity has been attributed to the ginsenosides. Ginsenosides are dammarane-type triterpene saponins and are common throughout the *Panax* species. There are four major groups of ginsenosides: dihydroxylated protopanaxadiol, trihydroxylated protopanaxatriol, ocotillol, and oleanic acid type, the basic structures of which are presented in Figure 1. 3 (Zhu et al., 2004). Compounds within these groups can be distinguished based on the number, type, and location of sugar moiety R-groups. Ginsenoside nomenclature follows the formula Rx, in which x can be a, b_1, b_2, c, d, e, f, g_1, g_2, g_3, h_1, h_2 or o_1, etc, named in order of position on thin layer chromatograms (Bahrke and Morgan, 2000). The major ginsenosides in North American ginseng are protopanaxadiol type Rb1, Rb2, Rc, and Rd, and protopanaxatriol type Re, and Rg1.

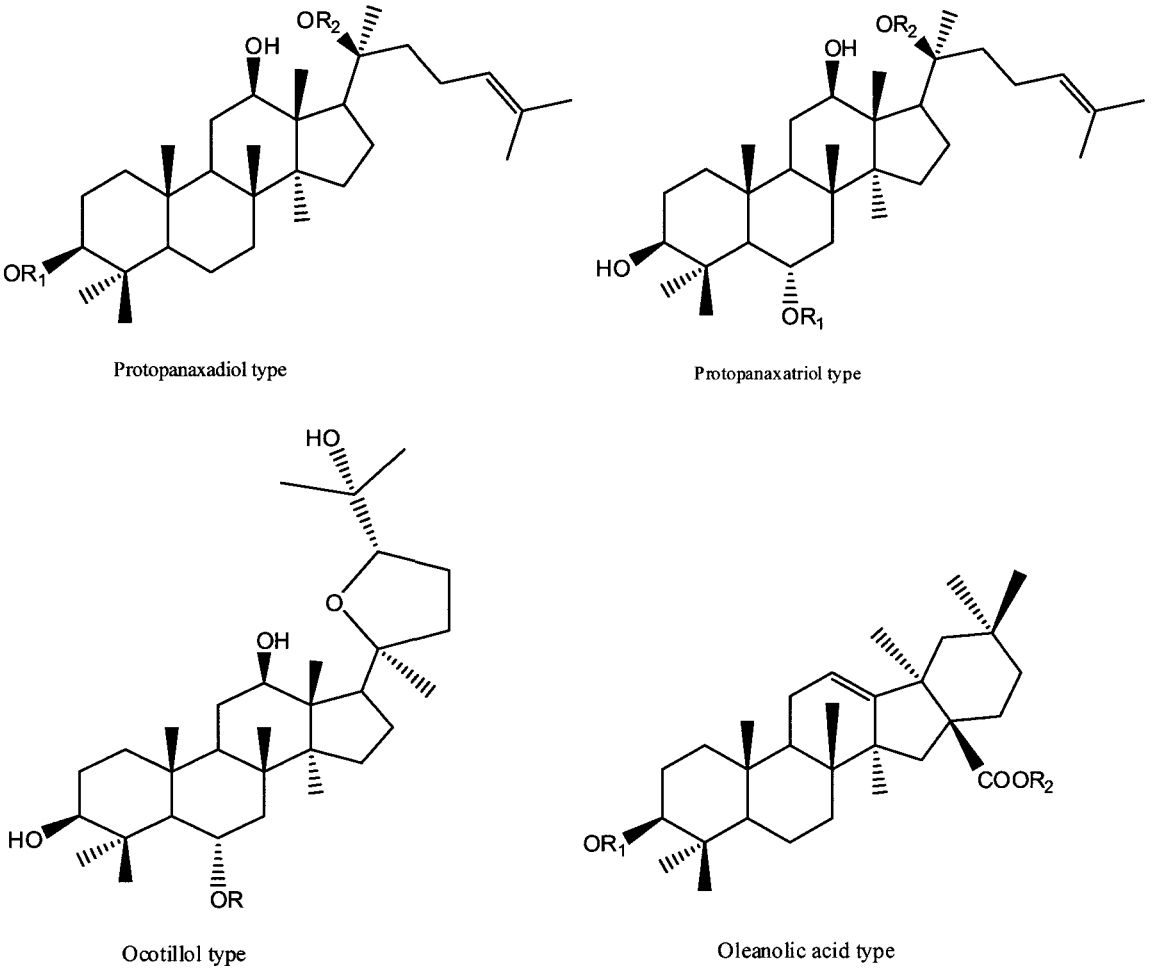

Figure 1. 3. Backbone structures of the 4 major ginsenoside groups.

1.2.4.2 Ginsenosides as markers

Over the past few decades, there has been interest in developing methods to conclusively distinguish the two main commercial ginseng species, North American *P. quinquefolius* and Asian *P. ginseng*. These ginseng species are similar in appearance yet have different traditional properties. Generally, *P. quinquefolius* is more expensive than *P. ginseng* but since these species are similar in appearance and often sold as powder or in slices, species identification and authentication can become an issue as mixing or mislabelling species can occur (Chan et al., 2000). Ginsenoside Rf and pseudoginsenoside

F11 have been studied extensively as marker compounds for *P. ginseng* and *P. quinquefolius* respectively. Rf and F11 have distinct molecular structures and are not convertible (Figure 1. 4) but have the same molecular formula ($C_{42}H_{72}O_{14}$); thus they are not easily separated under all chromatographic conditions (Li et al., 2000). Although Rf has been reported in low quantities in *P. quinquefolius* (Wang et al., 1999), a thorough LC-MS examination by Li et al. (2000) determined that Rf and F11 have distinct MS fragmentation patterns and during ionization, there are unique precursor and product ions. In the LC separation, F11 eluted approximately 1 minute prior to Rf. This study and others since have distinctly determined that Rf is absent in American ginseng and present in Asian ginseng and F11 is absent in Asian ginseng but present in American ginseng (Chan et al., 2000;Li et al., 2000;Wan et al., 2007;Li and Fitzloff, 2002). This is one way in which ginsenosides can be used as marker compounds with quality control implications.

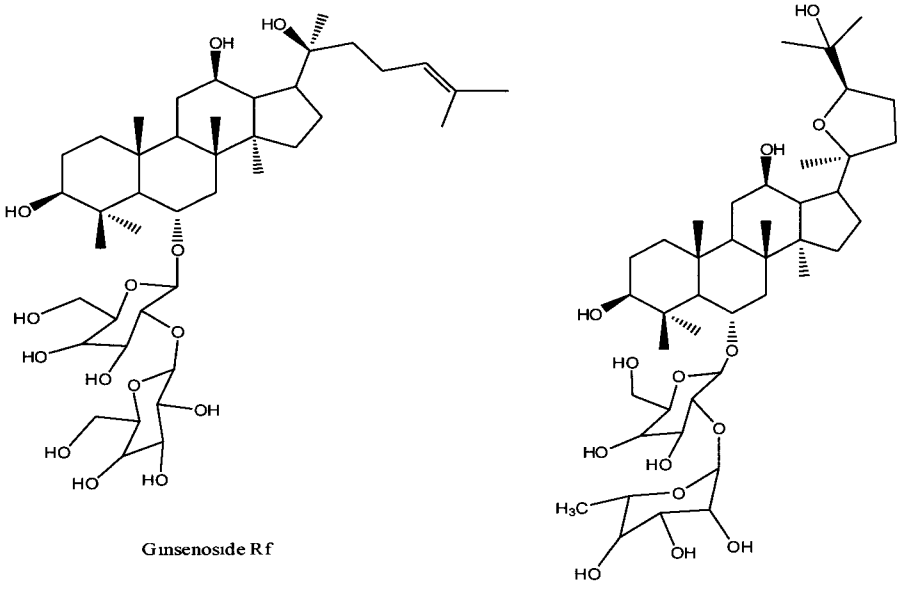

Figure 1. 4. Molecular structures of the Rf and F11, unique to *P. ginseng* and *P. quinquefolius* respectively.

Along with examining marker compounds, relative ratios of major ginsenosides are often used as species indicators. Several studies report the ratio of Rg1: Rb1 to be 1 or slightly greater in Asian ginseng (Zhu et al., 2004;Wang et al., 1999;Wan et al., 2007;Ji et al., 2001;Choi, 2008;Li et al., 2005). *P. quinquefolius* is thought to be distinct in its low Rg1:Rb1 ratio (Wan et al., 2007;Ji et al., 2001;Choi, 2008;Chen et al., 2007;Ligor et al., 2005;Assinewe et al., 2003;Wang et al., 2005). It was also reported that the ratio of Rg1:Re is specific for these species as the ratio of Rg1:Re was found to always be greater than 1 in Asian ginseng and less than 1 in North American ginseng (Chan et al., 2000). However, the use of these ratios as distinctive species markers was challenged as a study by Schlag and McIntosh (2006) who observed some Maryland populations of North American ginseng with Rg1:Rb1 ratio greater than 1 as well as two distinct chemotypes based on Rg1:Re ratios. In this study a Northern chemotype with a low Rg1:Re ratio was identified as well as a Southern chemotype with a high Rg1:Re ratio. This demonstrates that although ginsenoside ratios may often be used to help identify species, due to variation within species, ratios are not as reliable as distinct marker compounds. Species marker compounds and methods to quickly and reliably distinguish between ginseng species and chemotypes are highly desirable for quality control and standardization purposes.

1.2.4.3 Factors influencing ginsenoside content

Ginsenoside content in ginseng roots is influenced by several factors including age, location of growth, and root part. In terms of age, ginsenoside content has been shown to gradually increase with root age. In a study by Qu et al. (2009), ginsenoside content in ginseng root gradually increased between 1 and 5 years of age. However, in a previous study

examining six major ginsenosides, Rc, Rb1, and Rb2 content was significantly effected by age, while age had no effect on Rg1, Re, and Rd content (Lim et al., 2005).

Growing location influences ginsenoside content and variation has been observed both within and among populations of cultivated and wild *P. quinquefolius*. Although no difference has been observed in overall ginsenoside content between wild and cultivated populations (Schlag and McIntosh, 2006;Assinewe et al., 2003) variation has been shown between and within individual ginseng populations from different locations. In a report by Schlag and McIntosh (2006), a total of 44 roots were assessed from 10 populations in Maryland to examine inter-population variation of total and individual ginsenoside content. Total ginsenoside content varied (0.85- 5.78%) as did ratios of individual ginsenoside between populations. Assinewe (2003) examined wild ginseng populations from Ontario, Quebec, Maine, Vermont, and Wisconsin and again found high variability in total ginsenoside content between wild populations. In a study by Lim (2005), wild ginseng plants were harvested and transplanted to two separate forest garden locations and allowed to grow for two years. Ginsenoside Rb1, Rc, and Rb2 content were shown to be significantly different between the two locations. The variability of ginsenosides within individual populations has also been demonstrated. Li (1996) examined ginsenoside variation within a single British Columbia population and found variation in total ginsenoside content though greater variability was observed in the ratios of individual ginsenosides.

Depending on the root part assessed, ginsenoside levels differ. A study by Christensen (2006) showed total ginsenoside levels to be greater in root hairs compared to lateral roots and main roots. This was also shown to be the case for individual ginsenosides

Re, Rb1, Ro, malonyl-Rb1, and malonyl Rc, whereas the general trend of main root < lateral root < root hairs was seen when examining Rc, Rb2, Rd, and malonyl-Rd content.

1.2.4.4 Polysaccharides

Several polysaccharides have been isolated from *P. ginseng* and *P. notoginseng* (Table 1. 1). These species contain starch-like polysaccharides, consisting of 3-branched α-D-(1,6)-glucans and 6-branched α-D-(1,4)-glucans as well as pectins which consist largely of galacturonic acid, galactose and arabinose with minor contributions of glucose, mannose, rhamnose, and glucouronic acid (Zhang et al., 2009). Although individual polysaccharides have not been well characterized in *P. quinquefolius*, monosaccharide components include glucose, galactose, arabinose, rhamnose, mannose, and fucose (Assinewe et al., 2002).

Table 1. 1. Polysaccharides identified in *P. ginseng* and *P. notoginseng*.

Species	Polysaccharides identified	Type	Reference
P. ginseng	ginsenan S-IA	Acidic	Tomoda et al., 1993
	ginsenan S-IIA	Acidic	
	ginsenan PA	pectin	
	ginsenan PB	pectin	
	arabinogalactan I	type II arabinogalactan/ arabinogalactan protein (AG/AGP)	Yu et al., 2010
	arabinogalactan 2	type II AG/AGP	
	rhamnogalacturonan I-1	Pectin	
	rhamnogalacturonan I-2	Pectin	
	rhamnogalacturonan I-3A	Pectin	
	rhamnogalacturonan I-3B	Pectin	
	rhamnogalacturonan I-4	Pectin	
	HM-homogalacturonan	Pectin	
	panaxans A- U	peptidoglycan	Choi, 2008
P. notoginseng	arabinan	Pectin	Zhu et al., 2005
	4-galactan	Pectin	
	acidic rhamnogalacturonan	Pectin	
	homogalacturonan	Pectin	
	heteroxylan	non-cellulosic	
	xyloglucan	non-cellulosic	
	mannan	non-cellulosic	
	arabinogalactan 2	type II AG/AGP	

1.2.4.5 Other phytochemical constituents

Other constituents in ginseng include polyacetylenes and phenolics. Ten cytotoxic polyacetylenes have been isolated from North American ginseng. Of the polyacetylenes isolated, panaxydol and panaxynol (also called falcarinol) are the most abundant (Fujimoto et al., 1991;Fujimoto et al., 1992). These compounds are not highly stable like ginsenosides and polysaccharides and their concentration in dried root is very low. Several phenolic

compounds have been identified as minor components of *P. quinquefolius* roots, including vanillic acid, ferulic acid, and p-coumaric acid (He et al., 2009).

1.2.5 Biological activity of ginseng

Ginseng has been shown to possess several therapeutic effects. Ginsenosides have been associated with anti-diabetic effects in clinical trials along with antioxidant, anti-inflammatory, and anti-cancer activities, among others *in vitro* and *in vivo* (Vuksan et al., 2000a;Vuksan et al., 2001;Dascalu et al., 2007;Kitts et al., 2000;Keum et al., 2000;Qiu et al., 2009;Leung et al., 2007;Lim et al., 1997;Rudakewich et al., 2001). Polysaccharides have shown immunomodulatory activity *in vitro* and in clinical trials (Assinewe et al., 2002;Biondo et al., 2008;Predy et al., 2006) and polyacetylenes have been investigated for their cytotoxic effect on leukemia cells (Fujimoto et al., 1991).

1.2.5.1 Anti-diabetic activity

Ginseng has recently sparked interest due to its anti-diabetic properties found in clinical trials and laboratory tests. Type 2 diabetes mellitus (T2D) is an increasing concern worldwide with the occurrence predicted to increase from 171 million in 2000 to 366 million in 2030 worldwide (World Health Organization, 2009). T2D is characterized by hyperglycemia, which is due to insulin resistance or lack of insulin production. Hyperglycemia and insulin resistance exert negative effects on all organs and cells and lead to many serious complications including atherosclerosis, retinopathy, neuropathy, and nephropathy (Ahmed, 2005). Although several pharmaceutical drugs are available for use in T2D patients, the effects do not address complications resulting from diabetes (Li et al., 2004). Furthermore, common adverse effects of synthetic drugs include hypoglycemia, lactic

acid intoxication, and gastrointestinal upset. It is therefore of interest to examine complementary treatments with minimal side effects, focusing on treatment of hyperglycemia and insulin resistance, as well as diabetic complications.

North American ginseng extracts have shown promising results in clinical trials as a potential complementary therapy in T2D (Vuksan et al., 2000a; Vuksan et al., 2000b; Vuksan et al., 2001;Dascalu et al., 2007). In non-diabetic patients, *P. quinquefolius* has been shown to effectively reduce plasma glucose levels in healthy individuals, at doses of 3 to 9 g when administered at intervals of 0, 10, 20, 40, 80, or 120 minutes prior to a glucose challenge (Vuksan et al., 2001;Dascalu et al., 2007;Vuksan et al., 2000b;Sievenpiper et al., 2003). Though this effect was not dose-dependant, administration at 40 to 120 minutes before the glucose challenge produced the longest lasting glucose lowering results (Vuksan et al., 2001;Vuksan et al., 2000b). A study examining the effects of *P. quinquefolius* treatment in patients with T2D showed a similar significant reduction in post-prandial glycemia (Vuksan et al., 2000a). Although these results are promising, different product batches do not always show reproducible results. Sievenpiper et al. (2003) found no effect of a different *P. quinquefolius* batch on plasma glucose or insulin levels (Sievenpiper et al., 2003). This batch had a 48% reduction in total ginsenoside content as compared to the previously mentioned studies. However, a subsequent trial of five different batches of ginseng showed reproducible reductions in post-prandial glycemia and insulinemia. In this study ginsenoside levels were greater than levels of the previous efficacious trials (Dascalu et al., 2007). This shows the potential value of *P. quinquefolius* as a complementary therapy in the treatment of T2D and shows the importance of examining ginsenoside content in relation to biological effects.

Along with clinical trial results, ginseng has shown potential for controlling factors leading to diabetic complications in *in vivo* and *in vitro* studies. For example, *P. ginseng* and *P. notoginseng* have shown anti-atherosclerotic effects in animal models (Hwang et al., 2008;Zhang et al., 2008), and *P. ginseng* and *P. quinquefolius* have been shown to reduce the output of urinary protein associated with nephropathy in rats (Hyun et al., 2007;Ki et al., 2006). Ginsenoside Re has shown protective effects against retinopathy (Cho et al., 2006) and *P. ginseng* has shown potential neuroprotective effects in a diabetic rat model (Lim et al., 2002).

Although several anti-diabetic effects of ginseng have been shown, little is known about the underlying mechanisms of activity. Increased formation of advanced glycation endproducts (AGEs) is one mechanism that is thought to contribute to many diabetic complications. AGE production is initiated via a reaction between a protein and reducing sugar (i.e. glucose), forming a Schiff base. This reaction is concentration dependent, thus increased under hyperglycaemic conditions (Smit and Lutgers, 2004). The Schiff base undergoes a series of rearrangements over several days to form a stable ketoamine Amadori product. The Amadori product breaks down to reactive α-dicarbonyl glyoxal intermediates which crosslink to form AGEs (Ulrich and Cerami 2001; Ahmed, 2005). AGEs accumulate on long-lived proteins at a greater rate under diabetic conditions and can effectively cross-link connective tissues, such as collagen, increasing its rigidity and inhibiting tissue remodelling (Smit and Lutgers, 2004;Ulrich and Cerami, 2001). Cross-linking of vascular tissues contributes to thickening of capillary basement membranes which is a problem in retinopathy, nephropathy and neuropathy (Ahmed, 2005). *P. ginseng* has been shown to

reduce the formation glycated hemoglobin under high glucose conditions *in vitro* (Bae and Lee, 2004) and has been shown to reduce AGE formation in renal tissue of diabetic rats (Hyun et al., 2007;Ki et al., 2006). Few studies have examined the effect of ginseng on AGE formation and *P. quinquefolius* has not been studied in this respect.

1.2.5.2 Antioxidant activity

Major oxidative stress, often caused by a reduction in antioxidants, can cause severe damage to body cells, including DNA and protein damage, and lipid peroxidation. Oxidative stress is implicated in a range of health concerns, including diabetes mellitus, cancer, cardiovascular disease, Alzheimer's disease, and Parkinson's disease among many other degenerative diseases (Singh and Singh, 2008;Chan et al., 2008). In the case of diabetes, oxidative stress is elevated under hyperglycaemic conditions and free radicals can contribute to the development of AGEs and diabetic complications (Rizzi, 2003). American ginseng does have documented antioxidant activity in *in vitro* assays (Kitts et al., 2000;Hu and Kitts, 2001). The antioxidant value of Ontario grown ginseng has not been assessed, nor has the effect of ginsenoside variability on antioxidant activity.

1.3 RATIONALE AND OBJECTIVES

Ginseng is Ontario's most important medicinal crop, and Ontario has become the largest grower of ginseng in North America. Asian *P. ginseng* has been well characterized phytochemically and its biological activities widely investigated. However, Ontario ginseng land races have not been extensively characterized. The overall objective of this thesis was to improve the characterization of Ontario ginseng, focusing on analysis of ginsenoside variation as well as the analysis of malonyl ginsenosides and monosaccharide components.

Distinguishing characteristics and methods to rapidly distinguish Ontario ginseng landraces were examined. Phtyochemistry was assessed in relation to anti-diabetic and antioxidant activities as well as drug-interaction potential. Specific chapter objectives were as follows:

1. To assess the ginsenoside variation within and between Ontario ginseng land races and Ontario ginseng breeding lines. Ginsenoside variation was assessed in relation to biological activities (Chapters 2 and 3).

Ontario ginseng has reached the land race stage and the properties that distinguish these land races have not previously been phytochemically defined. Ginseng breeding lines were created using micropropagation and ginsenoside content was assessed in comparison to 4 year old field crops of ginseng. This has potential implications in shortening the time required to obtain ginseng with a desirable amount of ginsenosides and in creating ginseng lines with unique ginsenoside profiles. Anti-diabetic and antioxidant activities and drug-interaction potential of ginseng were assessed in relation to phytochemical variation to assess whether or not ginsenoside profiles may relate to activity.

2. To characterize malonyl ginsenoside content and monosaccharide composition in Ontario ginseng samples (Chapter 4).

Malonyl ginsenosides and the carbohydrate portion of Ontario ginseng have not been well defined. As these components may possess important biological activities, there was interest in characterizing them in Ontario ginseng.

3. To differentiate Ontario ginseng landraces and ginseng species using NMR spectroscopy as a metabolomic tool (Chapter 5).

There is a demand for methods to rapidly and accurately distinguish between sources of natural products for quality control and standardization purposes. NMR was investigated as a metabolomic tool to rapidly distinguish between ginseng landraces and species.

CHAPTER 2

PREFACE

The work presented in this chapter examines ginsenoside variation in Ontario ginseng land races. Ginseng land races have not been previously assessed and the long history of production in Ontario gave us the opportunity to examine land races that have been growing separately from each other and wild populations for several decades. This project was conducted in collaboration with the Ontario Ginseng Growers Association, who gave us access to Ontario ginseng roots from farms that had a history of quality production and had been producing ginseng for over 50 years. Dan Brown and his team at Agriculture and Agri-food Canada collected the roots. I prepared the root samples, with extraction assistance from Alice Luu, and carried out the HPLC analysis and bioassays, with assistance from Cathy Sun on the anti-glycation assay. A manuscript version of this chapter has been accepted for publication in *Recent Advances in Phytochemistry*.

2.0 GINSENOSIDE VARIATION IN ONTARIO GINSENG LAND RACES AND BREEDING LINES- RELATING PHYTOCHEMISTRY TO ACTIVITY

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ABSTRACT

Variation in ginsenoside content in Ontario-grown North American ginseng (*Panax quinquefolius*) was evaluated within and between five Ontario ginseng land races using HPLC-DAD analysis. All ginsenosides, except Rg1, varied significantly between at least two land races and total ginsenoside levels were reduced in one land race. The greatest range in total ginsenosides observed within an individual land race was 4.36-16.61 % w/w. Ginsenoside variation was also observed in micropropagated Ontario ginseng lines, suggesting the potential for the development of unique breeding lines and cultivars. Anti-glycation and antioxidant activity of Ontario ginseng was also evaluated and assessed in relation to ginsenoside content and total phenolic content. Low inhibition of advanced glycation endproduct formation was observed. Radical scavenging activity was seen in the DPPH assay and this activity was correlated with both total ginsenoside and total phenolic content. This activity was low in comparison to previously tested phenolic-rich extracts. Identifying variation in ginsenoside profiles and this effect on biological activity could be important for quality control purposes as well as land race and clonal breeding line development.

2.1 INTRODUCTION

Cultivated North American ginseng (*Panax quinquefolius* L., Araliaceae) is one of North America's most valuable medicinal crops, worth an estimated $100 million annually, and it is also one of the top selling dietary supplements in the United States (Mihalov et al., 2000;Small and Catling, 2003). Ontario has recently become the largest grower of North American ginseng with over 5000 acres in production (Ontario Ginseng Growers

Association, 2009). Ginseng is commercially important in Canada and the United States and as an export to Asian markets (Ontario Ginseng Growers Association, 2009).

In Traditional Chinese Medicine, American ginseng is used to treat a "yin" deficiency, or to replenish vital energy. Ginseng has been examined for a wide array of activities, including anti-diabetic, anti-cancer, and antioxidant activities, and immunostimulation (Assinewe et al., 2002;Kitts et al., 2007;Dascalu et al., 2007;Vuksan et al., 2000a;Keum et al., 2000) and it contains several bioactive metabolites, including ginsenosides, polysaccharides, and polyacetylenes. Ginsenosides have received the most attention as they are commonly believed to be responsible for the majority of ginseng's biological activity. The major ginsenosides in *P. quinquefolius* are Rb1, Re, Rd, Rg1, Rc, and Rb2. Roots have been the focus of study due to their high ginsenoside levels, traditional use, and high commercial value.

The amount of ginsenosides present in roots varies due to several factors including plant age, time of harvest, and location (Schlag and McIntosh, 2006;Lim et al., 2005;Assinewe et al., 2003;Dong et al., 2003). Variation in ginsenoside profiles between different populations of the same ginseng species can result in distinct chemotypes with unique ginsenoside composition. For example, a recent study of ginseng populations in New York State reported some populations with higher Rg1 than Re content, the opposite of previous reports (Lim et al., 2005). An inverse relationship between Rg1 and Re was observed where when Rg1 content was low, Re content was high and when Re content was high, Rg1 content was low. Schlag and McIntosh (2006) later identified two distinct chemotypes, a low Rg1/ high Re Northern and a high Rg1/ low Re Southern chemotype. The

study of ginsenoside variation is of interest particularly due to the potential identification of unique ginsenoside profiles and chemotypes. This could be important for quality control standards, as well as land race and clonal breeding line development.

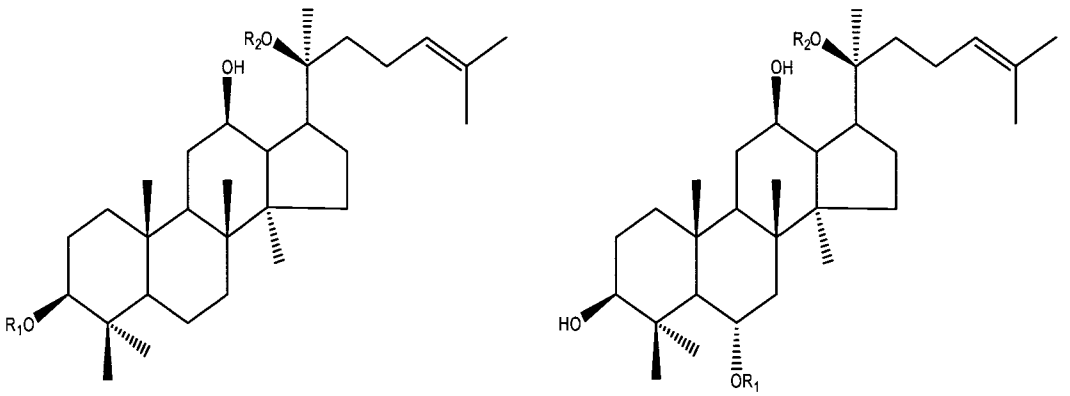
Ontario ginseng cultivation began over 100 years ago with the Hellyer family in Norfolk County (Ontario Ginseng Growers Association, 2009). Seed was originally obtained from wild ginseng populations and cultivation in Ontario has grown to include close to 400 growers. Today Ontario ginseng consists of several unimproved land races (Bai et al., 1997;Schulter and Punja, 2002), which are farmer selected varieties that have likely diverged significantly from wild populations, developing separately for several decades. Land races contain less genetic diversity than wild ginseng populations, however, are broader in genetic diversity than cultivars and clonal breeding lines.

Ginseng is a very high investment crop, requiring three to four years of growth before harvest. Seeds do not develop until about 3 years and require 12-18 months of stratification before germination (Zhou and Brown, 2006). There is interest in developing propagation techniques by which the same levels of ginsenosides can be obtained in roots within a shorter time period. Micropropagation has the advantages of reducing the generation cycle time by approximately 80% (Zhou and Brown, 2006), growing plants in a pathogen free environment, and allows for the potential to develop clonal breeding lines. This could lead to the development of Ontario ginseng lines with predictable quality, safety, and medicinal properties.

Ginseng has several reported biological activities which are attributed to ginsenoside content. One reason for assessing ginsenoside variation is that variation in active constituents may lead to variation in biological activity. For example, in clinical trails with both non-diabetic participants and patients with well controlled type 2 diabetes, American ginseng was shown to significantly reduce post-prandial glycemia (Vuksan et al., 2000a;Vuksan et al., 2000b;Vuksan et al., 2001). However, in a subsequent trial no significant reduction was found and this ginseng batch was shown to have a 48% reduction in total ginsenoside content compared to the initial effective batch (Sievenpiper et al., 2003). This demonstrates the importance of considering phytochemical profile when assessing biological activity.

Ginseng has been reported to have anti-diabetic and antioxidant activities. One important underlying mechanism responsible for diabetic complications is the elevated formation of advanced glycation endproducts (AGEs). AGEs are formed when a free amino group of a protein reacts with a reducing sugar (i.e. glucose or fructose) through a non-enzymatic nucleophilic addition reaction. AGE formation is increased under hyperglycemic conditions and they lead to cross-linking of proteins, tissue and cellular damage (Ahmed, 2005). Reducing AGE formation could be a potential target for reducing diabetic complications. The Asian species, *Panax ginseng* has been shown to reduce AGE formation (Bae and Lee, 2004), however, North American *P. quinquefolius* has not been assessed in this context. Oxidative stress is implicated in a range of health concerns, including diabetes mellitus, cancer, cardiovascular disease, Alzheimer's disease, and Parkinson's disease among many other degenerative diseases (Singh and Singh, 2008). *In vitro* antioxidant activity has been reported for North American ginseng (Kitts et al., 2000) however, it has not been assessed in relation to phytochemical constituents.

This study examines the variation of six major ginsenosides (Rg1, Re, Rb1, Rc, Rb2, and Rd) (Figure 2. 1) within and between five Ontario land races. To examine the potential for breeding line development using micropropagation, ginsenoside content in 4 year old seed derived roots (4YS) was compared to 4 year old micropropagated roots (4YM) and 1 year old micropropagated roots from clonal lines (1YM). As ginsenosides have been associated with anti-diabetic and antioxidant activities, the effect of ginsenoside variation on these activities is discussed. This is one step toward improving the phytochemical and biological characterization of Ontario ginseng.



	Ginsenoside	R_1	R_2
A	Rb1	-glucose^2-glucose	-glucose^6-glucose
	Rb2	-glucose^2-glucose	-glucose^6-arabinose (pyranose)
	Rc	-glucose^2-glucose	-glucose^6-arabinose (furanose)
	Rd	-glucose^2-glucose	-glucose
B	Re	-glucose^2-rhamnose	-glucose
	Rg1	-glucose	-glucose

Figure 2. 1. Chemical structures of 6 major ginsenosides.

2.2 MATERIALS AND METHODS

2.2.1 Material

For the land race analysis, twenty-one to twenty–five four-year old roots were collected from 1m x 1m plots from five ginseng farms in Norfolk County, Ontario. The GPS coordinates of these farms are as follows:

43 08 13.05 N, 80 24 06.10 W

43 02 48.93 N, 80 23 45.15 W

42 55 43.95 N, 80 24 19.53 W

42 48 10.13 N, 80 31 55.20 W

42 51 12.64 N, 80 32 27.05 W

For the breeding lines study, all lines originated from the same farm. 4YS roots were obtained from a single ginseng farm in Norfolk county Ontario. 4YM and 1YM were micropropagated from seedlings from the same farm according to the method by Zhou and Brown (Figure 2.7, Appendix) (Zhou and Brown, 2006). 4YM roots were micropropagated from seeds and 1YM lines were 1 year clonal lines from seeds from the same source. Ten 4YM, ten 4YS, and fifteen 1YM roots were assessed.

2.2.2 Extraction

Root sections with diameter between 4-10 mm were examined in the five land races, 4YS, and 4YM lines. 1YM roots were between 1-2 mm in diameter. 400 mg of ground root was added to 10 mL of 70% methanol and sonicated for 25 minutes at room temperature, centrifuged and the supernatant removed. The extraction was repeated twice using 10 mL and then 4 mL. The phases were combined and brought to 25 mL. For HPLC analysis, 100

µL of 5% potassium hydroxide (KOH) was added to 1mL of extract and incubated in the dark for 2 hours. The extract was then neutralized with 14% potassium dihydrogen phosphate (KH_2PO_4) and filtered through a 0.2 µm PTFE filter (Chromatographic Specialties Inc., Brockville, ON, CAN). For assays, the supernatant was dried using a speed vac and lyophilized.

2.2.3 HPLC- DAD analysis

All solvents were HPLC-grade and purchased from Fisher Scientific (Ottawa, ON, CAN). Pure ginsenoside compounds (Rg1, Re, Rb1, Rb2, Rc, and Rd) were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ, USA). A standard mix of pure ginsenosides was kindly provided by Dr. Anthony Windust (NRC, Ottawa, CAN). Samples were analyzed using a validated HPLC-DAD method developed by Paula Brown (BCIT, BC, CAN) and the major ginsenosides Rg1, Re, Rb1, Rc, Rb2, and Rd were quantified. HPLC analysis was conducted on a Hewlett-Packard Chemstation series 1100 chromatograph (Agilent, Palo Alto, CA, USA) consisting of an autosampler, quaternary pump, and a diode array detector (DAD). A Phenomenex Luna C18 column (150mm x 4.6mm; 5µm particle size; 100A pore size) was kept at 25°C and a flow rate of 1.5 mL/min maintained. The mobile phase consisted of water (HPLC grade) (A), 80:20 acetonitrile: water (B), and acetonitrile (C). Initial conditions of 76% A: 24% B were held isocratically for 8 minutes then changed following linear gradients to 68% A: 32% B in 18 minutes, 60% A: 40% B in 25 minutes, and 52% A: 48%B in 42 minutes. The column was then washed with 100% C over the next 11 minutes and returned to initial conditions for a total run time of 53 minutes. The column was allowed to re-equilibrate at initial conditions for 7 minutes between samples. 10 µL of sample was injected and the profile was monitored at 203 nm.

2.2.4 Anti-glycation activity

To determine the potential of American ginseng to inhibit AGE formation, a fluorescence based *in-vitro* assay was conducted. This assay was carried out as described by Farsi et al. (2008) with modifications (McIntyre et al., 2009). Stock solutions of glucose (200 mM)/fructose (200 mM) and bovine serum albumin (BSA; 2 mg/mL) (Sigma, St. Louis, MO, US) were prepared in 100 mM sodium phosphate monobasic monohydrate buffer (pH 7.4) (EM Science, Darmstadt, Germany). The solutions were filtered through a sterile 0.2 μm Nalgene filter unit (Nalge Nunc International, Rochester, NY, US) prior to use.

Incubation media containing BSA (1mg/ml), glucose (100 mM)/ fructose (100 mM), and vehicle, or standard compound (quercetin) were prepared. Experimental treatments consisted of BSA, glucose/fructose, and extract. Several extract concentrations were tested in attempt to determine a dose dependant response. A BSA blank was included in the assay (1 mg/mL in 100mM sodium phosphate buffer with vehicle) to control for inherent fluorescence of BSA. A sodium phosphate buffer blank was also incorporated. An extract blank was included consisting of glucose (100 mM)/ fructose (100 mM) in sodium phosphate buffer and extract or quercetin to control for any fluorescence produced from the sample itself. A positive control consisting of glucose (100 mM)/ fructose (100 mM), BSA (1 mg/mL), and 10 μg/mL of quercetin prepared in ethanol/water (80%:20%) in sodium phosphate buffer and a negative control of glucose (100 mM)/ fructose (100 mM), BSA (1 mg/mL), and vehicle in sodium phosphate buffer were tested. Four replicates of 200 μL were transferred into wells of sterile opaque polystyrene 96-well clear bottom plates (Corning Inc.,

Corning, NY, US). Plates were covered, sealed with parafilm, and incubated for 7 days at 37°C while shaking.

Following incubation, fluorescence was measured using a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, US) at excitation and emission wavelengths of 355 nm and 460 nm. The average fluorescence reading of the BSA blank was subtracted from each fluorescence reading of the experimental ($F_{experimental}$) and negative control ($F_{negative}$) treatments. The average fluorescence reading of the phosphate buffer blank was subtracted from each reading of the extract blank treatment ($F_{extract\ blank}$). The average $F_{extract\ blank}$ was subtracted from each corresponding $F_{experimental}$ resulting in corrected experimental treatment fluorescence values ($F_{experimental\ corrected}$). Percent inhibition of AGE formation was determined according to the following calculation:

Percent inhibition= $[(F_{negative} - F_{experimental\ corrected})/ F_{negative}]*100\%$ (Farsi et al., 2008)

The concentration inducing 50% AGE inhibition was determined and average IC_{50} values were calculated based on three replicates unless otherwise noted.

2.2.5 Antioxidant activity

As phenolics are well known antioxidant agents, the Folin-Ciocalteau total phenolics assay was performed to assess phenolic content in ginseng land race samples. The Folin-Ciocalteau reducing assay was performed as described by Singleton and Rossi (1964) with modifications by Harris et al (2008). A stock solution of 7.5% $NaHCO_3$ in distilled water was prepared and Phenol reagent was purchased from BDH Inc. (Toronto, ON, CAN.

Extracts were dissolved in 70% methanol and tested at 5 mg/ml, the experimentally determined optimal concentration for this assay. Quercetin prepared in 70% methanol was used in this assay to generate a standard curve based on the absorbance at 5 concentrations. 160 µL of extract or standard solution was added to 800 µL of Folin reagent. The mixture was then vortexed briefly and left to stand at room temperature for 5 minutes. 540 µL of the $NaHCO_3$ solution was added to the mixture and gently stirred. Samples were transferred to a clear bottom non-sterile 96-well plate (Nalge Nunc International, Rochester, NY, US) in three replicates of 200 µL/well. The plate was covered and incubated at room temperature in the dark for two hours. Absorbance was read at 725 nm and total phenolic content was calculated in terms of mg quercetin equivalents/ mg extract.

The DPPH assay, which tests radical scavenging, was performed as described by Harbilas et al. (2009) with incubation time increased to 65 minutes, the experimentally determined optimal incubation time (Figure 2.8, Appendix). DPPH was dissolved in methanol at a concentration of 100 µmol/L. Extracts dissolved in 70% methanol were tested at final concentrations of 2.5, 1.9, 1.4, 1.05, 0.79, and 0.59 mg/mL. For the experimental treatment, 250 µL DPPH solution was added to 40 µL of extract (tested at 6 concentrations) in a microplate well. A blank with only methanol was included as well as a positive control (ascorbic acid) which was tested at 6 concentrations to generate a standard curve for comparison. Absorbance was read with a microplate reader at 517 nm. The percent radical scavenging was calculated according to the following formula:

%Radical Scavenging= ($Absorbance_{(experimental)}$- $Absorbance_{(blank)}$)/ $Absorbance_{(experimental)}$ x 100

(Harbilas et al., 2009)

The inhibitory concentration for 50% scavenging (IC_{50}) was determined according to the linear regression model. This value was then compared to the IC_{50} generated from the ascorbic acid standard curve to determine DPPH radical scavenging activity:

DPPH activity= log IC_{50} ascorbic acid/ log IC_{50} sample

2.2.6 Statistical analysis

Ginsenoside content is expressed as ginsenoside weight relative to dry root weight (% w/w). For land race analysis, ginsenoside content data were not of normal distribution, therefore the Kruskal-Wallis test was used to determine differences in ginsenoside content between populations. For breeding line analysis data was normally distributed and an ANOVA test and Tukey's post-hoc test was used to determine differences in ginsenoside content between land races. Results were considered significant at $p<0.05$. Linear regression was used to assess the correlation between ginsenoside or phenolic content and biological activity.

2.3 RESULTS AND DISCUSSION

2.3.1 Land race variation

A typical chromatogram from one land race root sample is presented in Figure 2. 2. Significant variation in the content of most ginsenosides was seen between land races, with Rg1 being the only ginsenoside not differing in quantity between at least two land races ($p>0.05$) (Table 2. 1). These results may reflect the different selection pressures on plants at individual farms, including environmental factors and differences in cultivation techniques. Total ginsenoside levels were significantly reduced in one land race (5.54% w/w) whereas

the other four land races did not significantly differ in total ginsenoside content (7.46-8.53% w/w). Ginsenoside composition was similar between land races with the following most common general trend: Rb1 > Rd ≈ Re > Rc > Rg1 > Rb2. Land race 5 was the only land race showing higher overall Re than Rd though this was not statistically significant. This profile is unique in comparison to American ginseng populations in other locations where Rd is typically one of the lowest contributing ginsenosides (Schlag and McIntosh, 2006;Lim et al., 2005;Assinewe et al., 2003) (Table 2. 2). The greatest range in total ginsenosides within an individual landrace was between 4.36-16.61 %w/w, and the lowest range was between 4.24-10.24 %w/w.

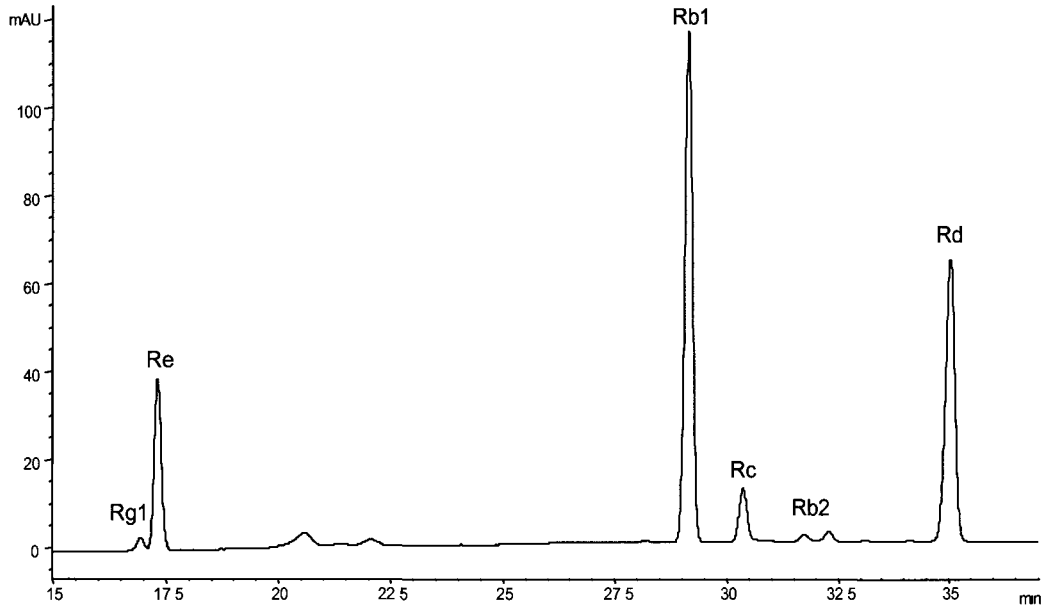


Figure 2. 2. HPLC-DAD chromatogram of one Ontario ginseng root sample.

Table 2. 1. Mean ginsenoside content (% w/w) (± SD) of roots from 5 Ontario land races (n= 21- 25). Significant differences between populations are indicated by different letters.

	Ginsenoside Content (% w/w) (± SD)						
Land race	Rg1	Re	Rb1	Rc	Rb2	Rd	Total
1	0.17 (0.08)^{a}	1.45 (0.58)^{a}	4.23 (1.36)^{ab}	0.66 (0.28)^{ab}	0.13 (0.05)^{a}	1.74 (1.08)^{a}	8.38 (2.80)^{a}
2	0.17 (0.08)^{a}	1.31 (0.41)^{a}	4.53 (0.85)^{a}	0.58 (0.23)^{ab}	0.11 (0.04)^{ab}	1.83 (1.09)^{a}	8.53 (2.11)^{a}
3	0.16 (0.09)^{a}	1.16 (0.30)^{ab}	4.21 (1.03)^{a}	0.44 (0.11)^{a}	0.11 (0.07)^{b}	1.39 (0.71)^{a}	7.47 (1.63)^{a}
4	0.18 (0.07)^{a}	1.40 (0.47)^{a}	4.45 (1.63)^{a}	0.63 (0.24)^{b}	0.12 (0.04)^{a}	1.59 (0.95)^{a}	8.38 (2.81)^{a}
5	0.17 (0.09)^{a}	0.99 (0.27)^{b}	3.09 (0.86)^{b}	0.46 (0.14)^{a}	0.12 (0.08)^{ab}	0.71 (0.32)^{b}	5.54 (1.43)^{b}

Our study showed mean total ginsenoside levels that were greater than mean levels reported in several other cultivation locations including New York State, Maryland, British Columbia, as well as wild populations in Ontario, Québec, Vermont, Maine, and Wisconsin (Table 2.2) (Schlag and McIntosh, 2006;Lim et al., 2005;Assinewe et al., 2003;Li et al., 1996). This may be due to the use of root sections in our study versus whole roots, though not all studies specified the root types used or root diameters. Roots ranging from 5-10 mm in diameter have been shown to contain higher levels of some individual ginsenosides than roots with a 15-38 mm diameter, though total ginsenoside levels were not significantly different between roots within this entire diameter range (Christensen et al., 2006). Along with the effect of cultivation location, it is also possible that differences in extraction methods, chromatographic conditions, and root age could account for the differences in ginsenoside levels.

Table 2. 2. Comparison of total ginsenoside content between different locations and cultivation methods.

Study	Mean ginsenoside content (%w/w)							Production method	Location
	Total	Rg1	Re	Rb1	Rc	Rb2	Rd		
This study	7 7	0 17	1 26	4 1	0 55	0 12	1 45	cultivated	Ontario
Li et al (1996)	3	0 18	1 1	1 22	0 18	0 02	0 29	cultivated	British Columbia
Assinewe et al (2003)	4 9	0 25	1 75	1 88	0 36	0 13	0 48	cultivated	Quebec
Assinewe et al (2003)	5 8	0 94	1 42	2 81	0 42	0 09	0 29	wild	Ontario, Quebec, Maine, Vermont, Wisconsin
Lim et al (2005)	2 7	0 8	0 6	0 95	0 2	0 05	0 1	wild and cultivated	New York State
Schlag et al (2006) (high Rg1/low Re)	2 3	1 04	0 25	0 69	0 23	n/a	0 05	wild and cultivated	Maryland
Schlag et al (2006) (low Rg1/high Re)	2 5	0 23	1 13	0 83	0 23	n/a	0 1	wild and cultivated	Maryland

More important than overall mean ginsenoside levels, it is significant that high variation is seen between land races in this study as well as between populations when compared to previous reports. A study of wild American ginseng populations collected in Ontario, Quebec, Maine, Vermont, and Wisconsin and one cultivated population in Quebec showed total ginsenoside levels did significantly differ among populations with the lowest at 2.73± 0.63 % w/w and highest content at 10.93± 1.96% w/w (Assinewe et al., 2003). This range is much greater than the range of total ginsenosides observed between land races in our current study, where total ginsenoside content ranged from 5.54- 8.53% w/w. This may be due to the wider age range and geographical distribution which would result in greater environmental variability. Furthermore, wild populations are expected to contain more genetic variation than cultivated land races. As was seen in this study, significant variation was also reported among and within ginseng populations in Maryland with variation in total ginsenosides ranging from 0.85- 5.78 % in wild roots and 1.04- 4.07% in cultivated roots (Schlag and McIntosh, 2006). In a study of cultivated ginseng in British Columbia, significant variation was seen between 9 fields, though Rb1, Rc, and Rd were the only individual ginsenosides which varied significantly (Li et al., 1996). The variation in ginsenoside levels between growing locations is important for quality control purposes as it cannot be assumed that North American ginseng obtained from different locations will contain similar ginsenoside content. Furthermore, in this study greater ginsenoside variation was seen within each individual land race than between land races. As ginsenoside levels can vary significantly within populations, it is important to assess multiple roots from each population to obtain an accurate estimation of ginsenoside content.

2.3.2 Breeding line variation

High variation in ginsenoside content was seen in each line (Table 2.4 A, Appendix), which is useful when selecting individual plants for breeding and to create clonal breeding lines. When comparing between micropropagated and field grown roots, there was no difference in individual or total ginsenoside levels between 4 year old seed derived (4YS) or micropropagated (4YM) ginseng roots (Figure 2. 3). This is expected as roots were of the same age and ginsenoside content has been shown to be influenced by age (Qu et al., 2009). This shows that roots produced by micropropagation and allowed to grow for the same amount of time as natural field populations have the same ginsenoside content. This may be useful in eliminating early challenges associated with ginseng cultivation and shows the potential for plants to be grown in greenhouse or bioreactor environments, eliminating influences from pests and environmental variation in natural field conditions. One year old micropropagated roots from clonal lines contained significantly lower levels of total ginsenosides (Figure 2. 3). This was due to its lower levels of the two generally most prominent ginsenosides, Rb1 and Re. Lower ginsenoside levels are most likely due to shorter growth time and much smaller root size. While roots of the 4YS and 4YM lines ranged in weight from 6- 45 g, roots in of the 1YM line weighed less than 1 g. Although levels of Rb2 and Rd were not significantly different between any breeding lines, Rg1 and Rc content was significantly higher in the 1YM roots. This may be important in developing ginseng lines to treat conditions for which Rg1 and Rc show particular promise in treatment. In *in vivo* and *in vitro* studies, Rg1 has shown particular promise as a neuroprotective agent (Radad et al., 2004;Wang and Du, 2009), in promoting neurotransmission (Liu et al., 2010), and in attenuating amyloid-β in Alzheimer's models (Shi et al., 2010;Wang and Du, 2009).

Ginsenoside Rc has recently been shown to enhance the uptake of glucose in myotubes, suggesting it may be an effective complementary anti-diabetic agent (Lee et al., 2010).

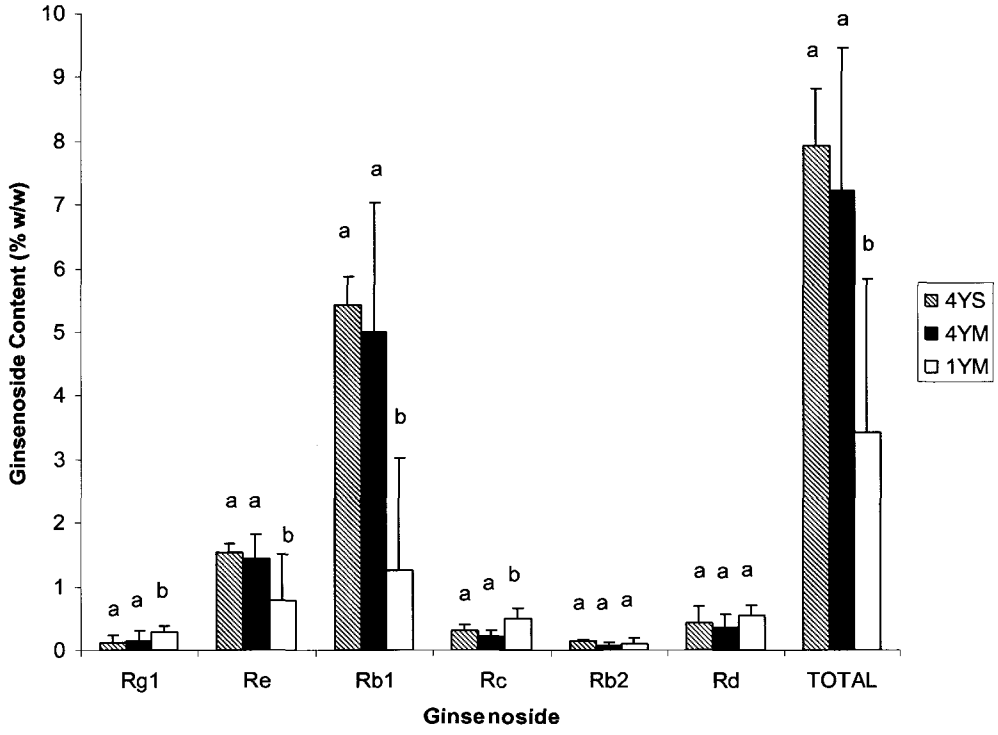


Figure 2. 3. Mean ginsenoside content (%w/w) in ginsenoside breeding lines. Different letters indicated significant differences as indicated by an ANOVA with Tukey's post-hoc test (<0.05).

Although ginsenoside levels were lower in the 1YM lines than the 4YS and 4YM lines, ginsenoside content was comparable to or slightly higher than levels previously reported in 4 year old roots. The 1YM lines contained total ginsenoside levels slightly greater than levels in 1 year old *P. quinquefolius* roots cultivated in China. Comparatively, Rb1, Rc, and Rd content were greater in the 1YM line, while Rg1 and Rb2 were similar between the two studies, and Re content was slightly lower in the 1YM lines (Qu et al., 2009). This

supports that micropropagation can produce ginsenoside levels similar to those in plants generated under normal field conditions.

The 4YS and 4YM lines had similar variation in ginsenoside profiles as can be seen in Figure 2. 4. Rb1 and Re were consistently the greatest contributing ginsenosides and the most common profile in both lines was Rb1 > Re > Rd > Rc > Rg1 > Rb2. One unique profile was observed in the 4YM lines which was not seen in any other line (Rb1 > Re > Rd > Rg1 > Rc > Rb2). A variety of ginsenoside profiles were also seen in the 1YM lines. While in most cases, Rb1 and Re were the highest contributing ginsenosides, in some roots Rd levels were greater than Re levels which was never the case in the other two lines. The most common profile of the 1YM lines was Rb1 > Re > Rc > Rd > Rg1 > Rb2 which was not very common in the 4YS and 4YM lines. Figure 2. 5 shows sample chromatograms from each of the lines. The unique profiles seen in some individual roots and the range of profiles represent an opportunity for unique clonal lines that could be produced in the future. These would be lines with consistent ginsenoside profiles and would contribute to quality control and standardization of ginseng products.

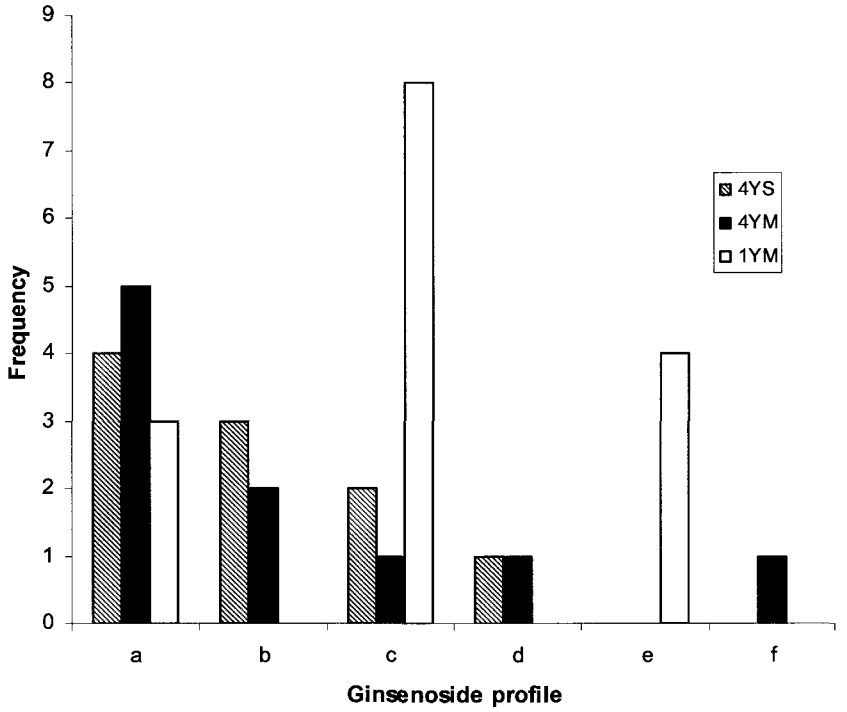


Figure 2. 4. Frequency of ginsenoside profile in each ginsenoside root line. Letters represent the following profiles (highest to lowest): a) Rb1, Re, Rd, Rc, Rg1, Rb2, b) Rb1, Re, Rd, Rc, Rb2, Rg1, c) Rb1, Re, Rc, Rd, Rg1, Rb2, d) Rb1, Re, Rc, Rd, Rb2, Rg1, e) Rb1, Rd, Re, Rc, Rg1, Rb2, f) Rb1, Re, Rd, Rg1, Rc, Rb2.

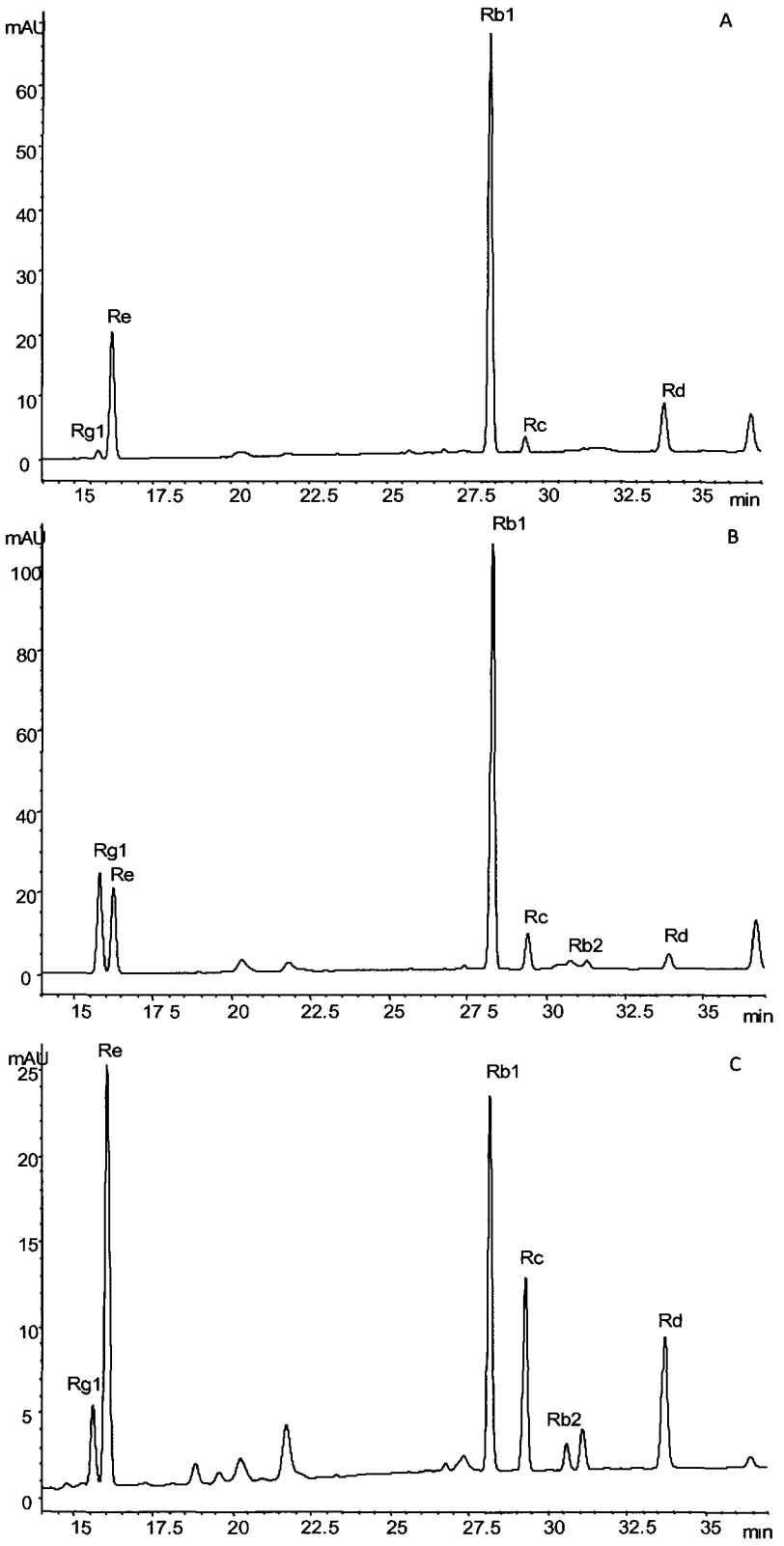


Figure 2. 5. Sample HPLC-DAD chromatograms from one a) 4 year old seed derived root, b) 4 year old micropropagated root, and c) 1 year old micropropagated root.

2.3.3 Anti-glycation and antioxidant activity of ginseng in-vitro

Ontario ginseng root extract was not a significant inhibitor of AGE formation. Inhibition did not reach 50%, with an extrapolated IC_{50} of >10 mg/mL (Table 2. 3). This value was high in comparison to extracts high in phenolic content such as lowbush blueberry, grape seed extract, and corn silk, which have IC_{50} values in the 1.3- 78.7 µg/mL range (Farsi et al., 2008;McIntyre et al., 2009;Sun et al., 2010), and the standard quercetin. Pure ginsenosides also had relatively low AGE-inhibition activity (Table 2. 3).

Table 2. 3. Inhibitory concentration 50% (IC50) of advanced glycation endproduct formation by pure ginsenosides and an Ontario ginseng extract (n=3).

Sample	**IC_{50}**
Rb1	1 – 10 mg/mL
Rd	1 – 10 mg/mL
Re	> 10 mg/mL
Rg1	0.5 – 1 mg/mL
Ontario ginseng extract	> 10 mg/mL
Quercetin	1.8 µg/ mL
Lowbush blueberry leaf extract	6.3 µg/ mL (McIntyre et al., 2009)
Lowbush blueberry stem extract	3.1 µg/ mL (McIntyre et al., 2009)
Grape seed extract	2.9- 16.5 µg/ mL (Sun, C., 2009)

Ontario grown American ginseng extracts had low total phenolic content in comparison to a number of previously tested plant extracts. Phenolic content in Ontario ginseng samples ranged from 0.67- 6.98 µg quercetin equivalents/ mg extract whereas values were in the order of 60- 269 µg quercetin equivalents/ mg extract for leaf extracts of plants

known to be rich in phenolic compounds (such as quercetin, catechin, and other flavonoids) (Harbilas et al., 2009). It is expected that roots would have lower phenolic content than leaves, which likely accounts for the relatively low phenolic content in ginseng roots.

Ontario ginseng samples produced IC_{50} values ranging from 1.35- 3.01 mg/mL in the DPPH radical scavenging assay. The biologically active concentration range was similar to that of a previously tested American ginseng extract (Kitts et al., 2000). IC_{50} values of samples were assessed in comparison to the IC_{50} for ascorbic acid and the relative activity was rather low. The maximum value for log IC_{50} ascorbic acid/ log IC_{50} ginseng sample was 0.14 which is quite low in comparison to other samples which are high in phenolic content, with this value ranging from about 0.5-1.1 (Harbilas et al., 2009). There was a correlation between DPPH radical scavenging activity and ginsenoside content (R^2=0.84) (Figure 2. 6a) and there was also a correlation between radical scavenging activity and total phenolic content (R^2=0.89) (Figure 2. 6b). In the case of ginseng the phenolic component is small but likely contributes to the antioxidant activity. Although ginsenoside levels are high, as illustrated above, in general, phenolic rich plants have tended to have greater antioxidant and anti-glycation activity than ginseng samples. It is possible that the antioxidant activity seen may be more related to phenolics than ginsenoside content.

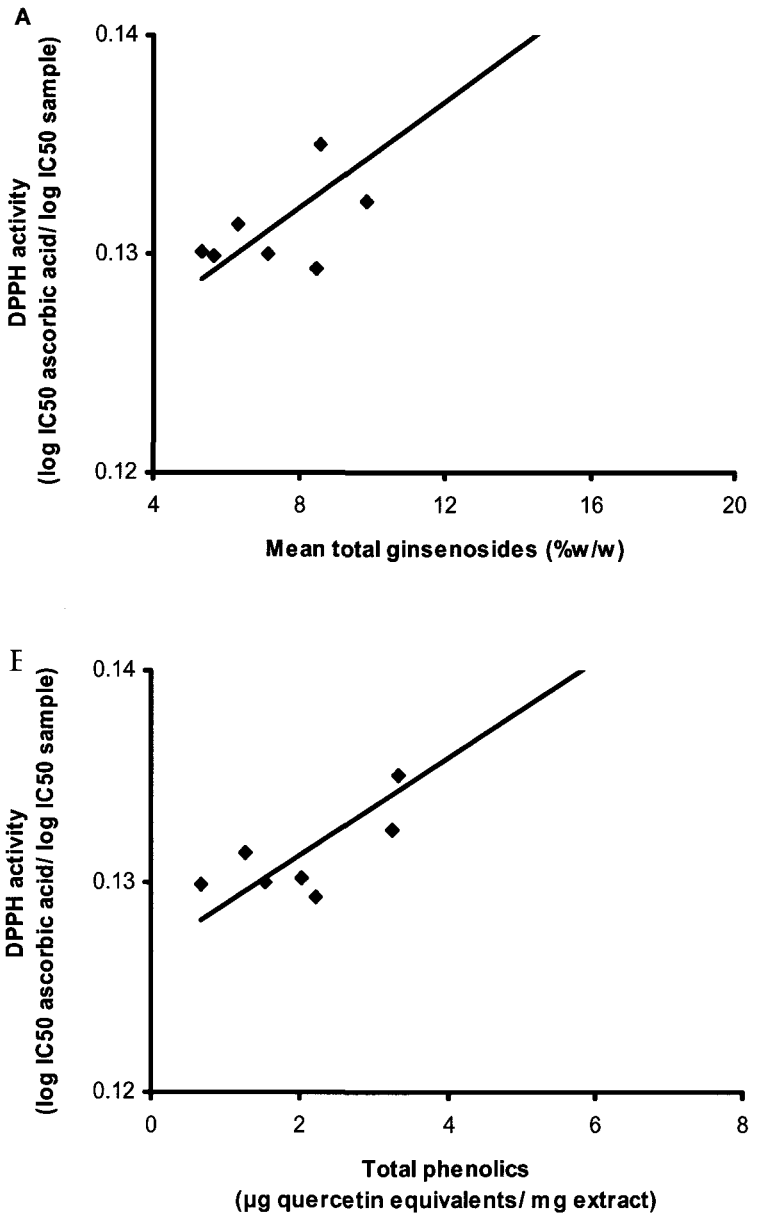


Figure 2. 6. (a) Radical scavenging activity as measured in the DPPH assay versus (a) total ginsenoside content (%w/w) and (b) Total phenolics (µg quercetin/mg extract) of eight ginseng root samples (n=3). The log IC_{50} of each sample was divided by the log IC_{50} of the standard ascorbic acid to serve as a comparison to the positive control.

Given the literature on anti-diabetic effects of ginseng, it was interesting that Ontario ginseng did not show strong anti-glycation and antioxidant effects *in vitro* as these activities are related to diabetic complications. It may be possible that ginseng acts by influencing glutathione in cells. Oxidative stress can lead to a reduction in glutathione levels and a study of ginsenoside Re administration to diabetic rats did result in increased glutathione (GSH) levels in the eye and kidney (Cho et al., 2006). In a study examining the effects of *Panax ginseng* on astrocyte cells under oxidative stress, glutathione reductase (which converts the oxidized glutathione disulfide (GSSG) to its normal reduced state) activity was significantly increased (Naval et al., 2007). To our knowledge, only one study has examined the effect of American ginseng on glutathione levels *in vivo*. In this study, American ginseng did not substantially improve glutathione regeneration in mouse hepatic tissue and also did not show significant *in vitro* antioxidant activity in the DPPH assay (Yim et al., 2002). Further investigation needs to be completed to determine the mechanism by which ginseng exerts antioxidant and anti-diabetic effects.

2.4 CONCLUSIONS AND FUTURE DIRECTIONS

This is the first attempt to examine family held ginseng land races which have been in cultivation for several decades. Clearly there is evidence of variation among these land races. Although HPLC-DAD analysis clearly quantifies ginsenoside levels and shows variability, it does not present any unique characteristics that would rapidly identify each land race. Nuclear Magnetic Resonance (NMR) methods are being developed in collaboration with Bruker BioSpin to differentiate ginseng landraces. This will allow for rapid quality control and standardization and may complement DNA fingerprinting. This will potentially provide a unique fingerprint for each population. As distinct land race characteristics are identified, it

is possible that these characteristics may be correlated with important activities. This could then lead to the development of unique, easily identifiable cultivars each with distinct biological uses.

Similar to the land races, significant variation in ginsenoside content was also found in micropropagated lines of ginseng root. This shows the potential for micropropagation to be used to develop clonal breeding lines. These lines could be selected for ginsenoside profile to create a variety of lines each with specific biological uses. Ginsenoside levels were similar between 4YS and 4YM lines as well as between the 1YM line and previously reported ginsenoside levels in 1 year old cultivated *P. quinquefolius*. This shows that ginsenoside levels can be reproduced using micropropagation. This would be of significant economical importance, potentially increasing harvest and reducing the initial input.

Although significant *in vivo* anti-diabetic effects have been reported, this did not translate into strong *in vitro* anti-glycation and antioxidant activities. There may be stronger *in vivo* antioxidant and anti-glycation activities which will be an important area of future investigation.

2.5 ACKNOWLEDGEMENTS

We would like to thank Paula Brown (British Columbia Institute of Technology) for developing, validating, and sharing the extraction and HPLC-DAD method. This project was funded by the Ontario Ginseng Innovation and Research Consortium.

2.6 APPENDIX

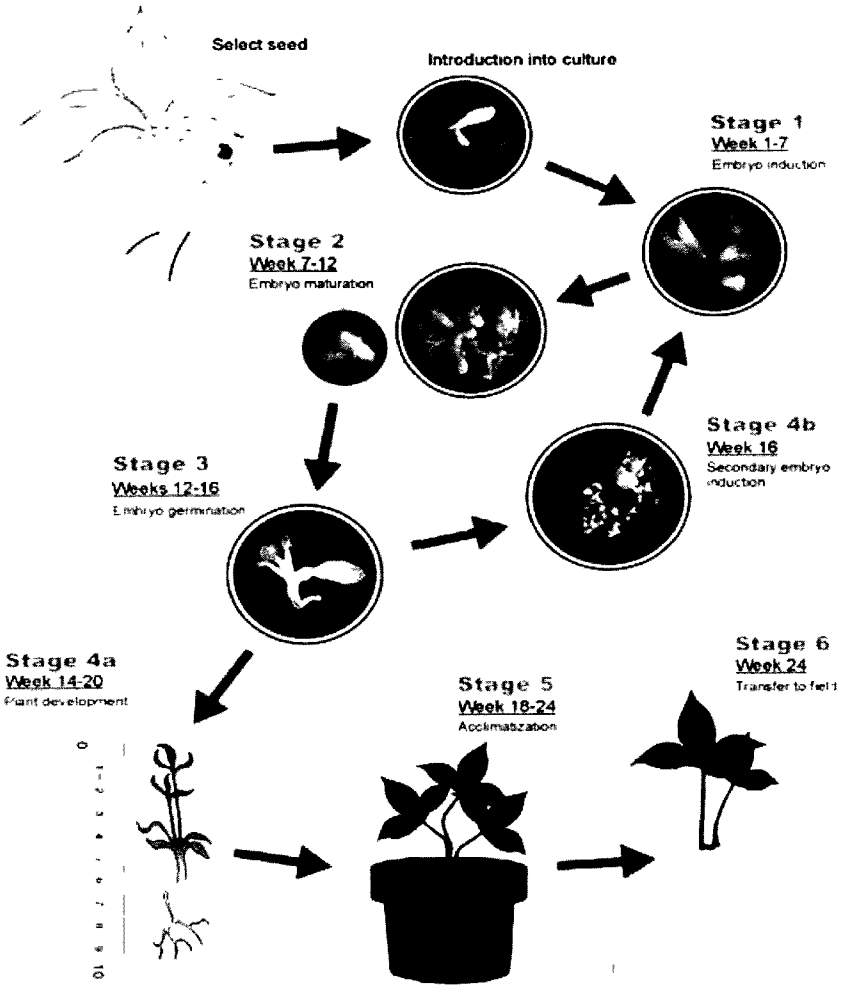


Figure 2. 7 A Flow-chart of the protocol for plant micropropagation of American ginseng from Zhou and Brown (2006).

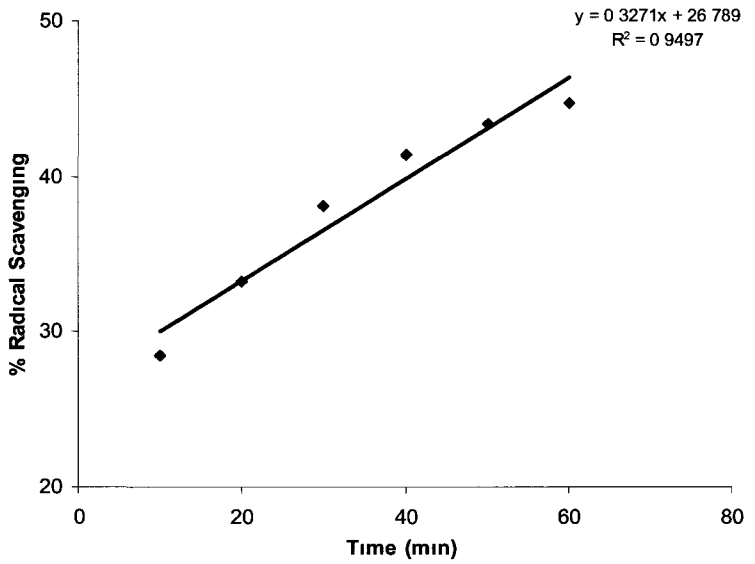


Figure 2 8 A Percentage of radical scavenging activity measured in the DPPH assay versus incubation time

Table 2 4 A Ginsenoside content (%w/w) in Ontario ginseng breeding lines

	Ginsenoside content (%w/w)						
Sample	**Rg1**	**Re**	**Rb1**	**Rc**	**Rb2**	**Rd**	**TOTAL**
4YS1	0 025937	0 520257	2 367933	0 085121	0 01997	0 259328	3 278547
4YS2	0 052295	2 29042	4 730371	0 354462	0 264328	0 482311	8 174186
4YS3	0 089111	1 979408	5 972234	0 633279	0 054367	0 32339	9 05179
4YS4	0 035878	1 20578	4 479344	0 318355	0 055161	0 513513	6 608033
4YS5	0 081797	0 995887	4 503603	0 23466	0 04292	0 442533	6 3014
47S6	0 055944	1 169089	4 076335	0 254162	0 086548	0 444072	6 08615
4YS7	0 229715	2 80304	6 31432	0 17491	0 163815	0 871873	10 55767
4YS8	0 316803	0 774957	6 863326	0 161007	0 134573	0 397804	8 648471
4YS9	0 078629	1 415196	8 79823	0 401476	0 315059	0 266963	11 27555
4YS10	0 245941	2 133787	6 050412	0 370443	0 21667	0 333215	9 350469
4YM1	0 550123	0 594256	4 111058	0 342799	0 156603	0 128195	5 883034
4YM2	0 057637	1 445746	3 822113	0 157745	0 104254	0 145046	5 732542
4YM3	0 082698	1 843992	4 964291	0 146932	0 103618	0 363058	7 504589
4YM4	0 156444	1 439908	4 027151	0 156128	0 029974	0 338799	6 148405
4YM5	0 072384	1 440953	4 189823	0 211566	0 030821	0 461681	6 407228
4YM6	0 112947	1 007715	4 843066	0 191809	0 042209	0 290843	6 488591
4YM7	0 195675	1 766963	4 613679	0 211496	0 037313	0 355301	7 180427
4YM8	0 089565	1 673745	4 709002	0 203911	0 035418	0 350059	7 0617
4YM9	0 063091	1 317949	10 6548	0 435266	0 081951	0 85927	13 41233
4YM10	0 12503	1 7804	3 96555	0 169933	0 030544	0 23086	6 302317
1YM1	0 120289	0 739814	0 891134	0 455929	0 070012	0 30174	2 578917
1YM2	0 315135	0 57238	0 871205	0 448581	0 068533	0 326602	2 602436
1YM3	0 242599	1 005017	1 175692	0 422858	0 07039	0 355331	3 271888
1YM4	0 24624	0 647486	0 849476	0 393637	0 068054	0 301556	2 506447
1YM5	0 381797	0 738678	0 895632	0 456015	0 078546	0 509641	3 06031
1YM6	0 287875	0 904931	1 475541	0 588526	0 100063	0 931327	4 288263
1YM7	0 274463	0 796173	2 379301	0 542052	0 101991	0 851111	4 945092
1YM8	0 424337	1 095435	1 863721	0 508414	0 087539	0 451723	4 431169
1YM9	0 189636	0 738943	0 896752	0 350495	0 058611	0 258476	2 492912
1YM10	0 225153	0 637796	0 80523	0 424941	0 076689	0 442656	2 612465
1YM11	0 305799	0 686223	1 227714	0 482069	0 082814	0 394413	3 179032
1YM12	0 178787	0 735367	1 295032	0 53704	0 098486	0 811764	3 656476
1YM13	0 231129	0 932634	1 621095	0 585989	0 111677	0 759629	4 242152
1YM14	0 595373	0 722577	1 637763	0 679706	0 130852	0 981874	4 748144
1YM15	0 144745	0 659683	0 936092	0 637046	0 103293	0 369119	2 849979

CHAPTER 3

PREFACE

This study was carried out primarily by Alice Luu, who was an undergraduate honours student in the Arnason lab, under my supervision and with my assistance. Alice conducted the CYP assays and I carried out the HPLC analysis. Teresa Tam also assisted with the CYP assays. This chapter combines Alice's honours thesis and manuscript under the same title which has been accepted in *Recent Advances in Phytochemistry*. The goal of this study was to examine the potential for ginseng to interact with drugs by influencing CYP3A4 and CYP2C9 enzymes and, building on Chapter 2, to examine the effect of ginsenoside content on activity.

3.0 PHARMACOGENETICS IN POTENTIAL HERB-DRUG INTERACTIONS: EFFECTS OF GINSENG ON CYP3A4 AND CYP2C9 ALLELIC VARIANTS

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ABSTRACT

This study focuses on the role of ginseng in potential herb-drug interactions through inhibition of cytochrome P450 enzymes CYP3A4 and three polymorphisms of CYP2C9. Examining commercial ginseng products and preparations made from cultivated ginseng roots, CYP3A4 inhibition correlated significantly with total ginsenoside content of the ginseng products tested but this correlation did not exist for CYP2C9 inhibition. The inhibition of CYP2C9 was low for the three allelic forms and the profile of inhibition by product did not vary with the three polymorphisms tested. These *in vitro* results suggest that CYP3A4 inhibition, but not CYP2C9 inhibition, may warrant further study in a clinical setting.

3.1 INTRODUCTION

Ginseng species (*Panax* spp.) have been used for centuries to treat a variety of ailments including fatigue, insomnia, impotence, and chronic illnesses. *Panax ginseng* C.A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (American ginseng) are the most commonly used and commercially grown ginseng species. Their pharmacological activities have been attributed to several bioactive components, of which the ginsenosides, which are triterpenoid glycosides, are the most important (Bahrke and Morgan, 1994).

In general, ginseng is not associated with any serious adverse effects if taken at recommended doses. However, there have been frequent reports of adverse reactions such as insomnia (Vuksan et al., 2008; Scaglione et al., 1996), gastric irritation (Engels et al., 2001; Allen et al., 1998), anxiety (Sievenpiper et al., 2006; Ellis and Reddy, 2002), and headaches (Lee et al., 2008; de Andrade et al., 2007). These adverse reactions are usually mild and

transient, with consumers recovering quickly after ceasing ginseng use. For this reason, ginseng has been regarded as generally safe and well-tolerated for the majority of users. In addition to adverse effects, there has also been growing concern for the potential of herb-drug interactions with the use of ginseng (Miller, 1998; Fugh-Berman, 2000).

Recently, Health Canada has indicated cautions for the concomitant use of *Panax ginseng* with warfarin, an anti-coagulant, and phenelzine, an anti-depressant and anxiolytic (Janetzki and Morreale, 1997; Shader and Greenblatt, 1985). A potential interaction has been observed between warfarin and *Panax ginseng* (Janetzky and Morreale, 1997; Rosado, 2002; Yuan et al., 2004; Lee et al., 2008) and although other studies have not been able to confirm such an interaction (Jiang et al., 2004), these unequivocal findings warrant further research in the effects of ginseng on warfarin pharmacodynamics and pharmacokinetics. Similarly, a potential herb-drug interaction has been observed with phenelzine (Shader and Greenblatt, 1988; Jones and Runikis, 1987; Shader and Greenblatt, 1985). These observations suggest that the administration of ginseng may affect the absorption, distribution, metabolism, elimination, and/or pharmacodynamics of other drugs.

Cytochrome (CYP) P450 enzymes are hemoproteins involved in the biotransformation of drugs and other xenobiotics ingested in the body (Chang, 2004). CYP3A4 and CYP2C9 are the major drug-metabolizing enzymes involved in the biotransformation of over 70% of clinically prescribed drugs. CYP3A4 is the primary drug-metabolizing enzyme involved in the biotransformation of more than 50% of clinically used drugs (Zhou et al., 2007). CYP2C9 is the major enzyme within the CYP 2C family and constitutes approximately 20% of human liver microsome content (Shimada et al., 1994). In

addition, it is also the second major contributor, following CYP3A4, to drug metabolism for commonly prescribed medications in the United States (Zanger et al., 2008). Ginseng could potentially inhibit or induce these enzymes, predisposing an individual to potential herb-drug interactions. Indeed, some studies have shown that ginsenosides, along with their metabolites, can inhibit CYP3A4 and CYP2C9 (Henderson et al., 1999; He and Edeki, 2004; Liu et al., 2006; Hao et al., 2008). Other studies, however, have not been able to reproduce these results. Etheridge et al. (2007), for example, did not find any significant alteration of enzyme activity using a ginseng root extract and ginsenoside metabolites in human liver microsomes (HLM) containing CYP3A4 and CYP2C9. Due to these conflicting results, it remains unclear whether the administration of ginseng could have a significant or physiologically relevant effect on CYP3A4 and CYP2C9 activity.

Pharmacogenetics is another important factor to consider during the assessment of herb-drug interactions. In the past, herb-drug interactions have only been examined using wild-type CYP enzymes with normal function. However, polymorphisms in the human genome are a common occurrence, leading to modified enzyme structure and/or function. Indeed, many polymorphisms have been identified in CYP genes, including those encoding CYP3A4 and CYP2C9. Interestingly, the CYP3A4 gene can tolerate many polymorphisms in the genetic code without compromising function. The gene for CYP2C9, however, is not as tolerant and polymorphisms often give rise to mutant alleles associated with clinical complications (Aithal et al., 1999).

Although over 50 polymorphisms have been identified in the CYP2C9 gene, only two allelic variants (CYP2C9*2 and CYP2C9*3) have been considered to be clinically relevant

and both variants harbor a single nucleotide mutation (Kirchheiner and Brockmoller, 2005). Compared to the wild-type allele (CYP2C9*1), the CYP2C9*2 allele consists of an Arg^{144} (CGC) to Cys^{144} (TGC) amino acid substitution. In the CYP2C9*3 allelic variant, the amino acid change occurs at Ile^{359} (ATT) to Leu^{359} (CTT). These alleles are most prominent in Caucasian populations and are associated with a decrease in enzyme activity when compared to the wild-type allele. Although research has examined the impact of CYP2C9 polymorphisms on drug metabolism, little is known about the potential of serious interactions when CYP2C9*2 or CYP2C9*3 are inhibited. Furthermore, there is currently no information on the effects of ginseng, one of the most widely used herbs, on CYP2C9 allelic variants.

Limited research has been conducted on CYP activity as compared to the natural ginsenoside profiles found in ginseng products. Furthermore, no research has been conducted on comparing the impact of different ginseng products and preparations on CYP3A4 or CYP2C9 activity. The main objective of this study was to determine the inhibitory potential of different ginseng roots extracts and commercial products on CYP3A4 and CYP2C9. The second objective of this study was to correlate ginsenoside profiles with inhibitory activity. Each ginseng product was subjected to HPLC analysis to determine the content and composition of six major ginsenosides (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd). This study has the potential to identify ginseng products that may potentially cause herb-drug interactions and to demonstrate the role ginsenosides may play in these interactions, which could have implications in clinical settings.

3.2 MATERIALS AND METHODS

3.2.1 Material and extraction

A collection of 4 ginseng roots and 4 ginseng commercial products were examined for CYP inhibition activity. Within the collection of ginseng roots, 3 were obtained from farms situated in Norfolk County, near Delhi Ontario. These Ontario roots were 4 years old with diameters ranging from 3-12 mm. HPLC analysis confirmed that these roots belonged to the *Panax quinquefolius* species. A methanol extract was made in-house while the ethanol and aqueous extracts were provided by Naturex (California, USA). A *Panax ginseng* root sample was also assessed, which was distributed by Trout Lake Farm after receiving it from China. The four commercial root products were assigned Natural Product Number (NPN) accession numbers and vouchers have been deposited in the herbarium at the University of Ottawa. To maintain company anonymity, the commercial products from local distributors were coded NPN1, NPN2 for *Panax ginseng* samples and NPN3, NPN4 for *Panax quinquefolius* samples.

Ground root and powdered commercial products were extracted in 70% methanol three times by sonication. Each phase was centrifuged and supernatants were pooled. For assays, the supernatant was dried in a CentriVap and lyophilized to obtain extract. For HPLC analysis, 100 µL of 5% potassium hydroxide (KOH) was added to 1mL of extract and incubated in the dark for 2 hours. The extract was then neutralized with 14% potassium dihydrogen phosphate (KH_2PO_4) and filtered through a 0.2 µm PTFE filter (Chromatographic Specialties Inc., Brockville, ON, CAN).

3.2.2 CYP3A4 Assays

The following solutions were used for CYP3A4 Studies:

a) Reaction buffer (0.5 M Potassium phosphate buffer – KH_2PO_4): A bulk solution of buffer was prepared using monobasic potassium phosphate (EMD Biosciences Inc, New Jersey, USA) and dibasic potassium phosphate (Fisher Scientific, Mississauga, ON). Each product was dissolved in distilled water to a final concentration of 0.5 M. After these solutions were prepared, monobasic potassium phosphate was mixed with dibasic potassium phosphate to make a 0.5 M reaction buffer. The pH of the buffer was 7.4.

b) NADPH – Reaction Catalyst*:* Reduced B-Nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich, location) was used to initiate the reaction with CYP3A4 (BD Gentest, Mississauga, ON). To prepare this solution, solid NADPH was dissolved in reaction buffer to a final concentration of 18 mM. This solution was mixed using a vortex. NADPH solutions were protected from light at all times.

c) Dibenzylfluorescein (DBF) – Substrate: A working solution of 200 μM DBF was made from a 10 mM stock solution of DBF (BD Biosciences, California) and vortexed. 20 μL of the stock solution was quickly withdrawn and dissolved into 980 μL of filtered acetonitrile (CH_3CN) in a 1.5 mL microtube. The working solution was vortexed and inverted several times to ensure mixing.

d) Ketoconazole – Positive control for CYP3A4 inhibition*:* A 1.88 mM stock solution of ketoconazole (Calbiochem, Mississauga, ON) was made by dissolving pure compound into 100% HPLC-grade methanol (Fisher Scientific, New Jersey, USA).

20 μL of the stock solution was then dissolved into 50 μL methanol, yielding a solution of 537 μM ketoconazole. Finally, 930 μL of distilled water was added to the solution until a concentration of 37.6 μM ketoconazole was attained. The concentration of methanol was adjusted to 7%. Two additional working solutions were prepared at 3.76 μM and 0.376 μM.

e) Reaction Solution 1, 2, 3: For the CYP3A4 inhibition assay, three reaction solutions were made according to Table 3. 1 below. These values were optimized for one single well in the assay. If multiple wells were screened for fluorescence, the values were multiplied by the number of wells tested in the assay. Reaction Solution 1 contained the substrate (DBF) along with the catalyst (NADPH). Reaction Solution 2 contained denatured enzyme while Reaction Solution 3 contained the active enzyme. Reaction Solution 2 served as a control for background fluorescence.

Table 3. 1. Composition of reaction solutions 1, 2, and 3 for the CYP3A4 bioassays.

Reagent	**Reaction Solution 1 (μL)**	**Reaction Solution 2 (μL)**	**Reaction Solution 3 (μL)**
Distilled water	43	65.7	65.7
Reaction buffer – 0.5 M	50	24	24
NADPH – 18 mM	6	-	-
DBF – 200 μM	1	-	-
Active CYP3A4 – 1 μM	-	-	0.3
Denatured CYP3A4 – 1 μM	-	0.3	-
Total	**100**	**90**	**90**

An Applied Biosensors Cytofluor 4000 Fluorescence Measurement System plate reader (MTX Lab Systems Inc, Virginia, USA) was used and adjusted to the following settings using the Cytofluor software: lamp on, filter at excitation 485/20, emission at 530/25, gain

50, mix time of 3 seconds, Costar 96 well plate, and total incubation time of 20 minutes with 3 cycles.

Reaction solutions 1, 2, and 3 were prepared with water and buffer and kept at room temperature. The assay plate was prepared using a vehicle control (7% methanol), a positive control (ketoconazole in 7% methanol), and test samples. For the vehicle and positive controls, 10 μL was directly aliquoted into the assay plate in triplicate. Based on a final in-well volume of 200 μL, the final concentration of vehicle and ketoconazole were 0.35% MeOH and 1.88 μM, respectively.

Methanol has been shown to interfere with CYP assays at high concentrations. Therefore, it was necessary to ensure a low methanol concentration in all wells. Since the ginseng samples were dissolved in 70% methanol, a dilution step with distilled water was required. 9 μL of distilled water was first plated, followed by the addition of 1 μL of extract. The final in-well concentrations of all tested samples were 20, 40, 80, 160, and 320 mg/mL. Once the assay plate was prepared, the environment was kept dark to protect NADPH from light exposure. All subsequent work was conducted in the dark with an ultraviolet lamp, unless otherwise specified.

Using the values listed in Table 3. 1, NADPH and DBF were vortexed and added to Reaction Solution 1. After mixing the solution thoroughly, 100 μL was aliquoted into all reaction wells. The final in-well concentration of NADPH and DBF were 0.54 mM and 1 μM, respectively. Reaction Solution 2 was then prepared by adding CYP3A4 (BD Biosciences, Mississauga, ON) previously denatured using a boiling water bath for 15

minutes. 90 μL of Reaction Solution 2 was aliquoted into the first set of vehicle, positive control, and test wells. Lastly, Reaction Solution 3 was made by adding active CYP3A4 (BD Biosciences, Mississauga, ON) previously thawed from a -80°C freezer. 90 μL of Reaction Solution 3 was aliquoted into the second set of vehicle, positive control, and test wells. The final in-well concentration of denatured or active enzyme was 1.5 nM in 0.185 M buffer. The assay plate was subsequently placed into the plate reader and scanned for fluorescence.

*3.2.3 CYP2C9*1, CYP2C9*2, CYP2C9*3 Assays*

The following solutions were used for CYP2C9 studies:

a) Reaction buffer (0.5 M TRIS buffer): A bulk solution of buffer was prepared using Tris base (Invitrogen Canada Inc, Burlington, ON) and distilled water. The pH of the buffer was adjusted to 7.5.

b) NADPH – Reaction Catalyst: Reduced B-Nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich, location) was required to initiate the reaction with CYP2C9 enzymes (BD Gentest, Mississauga, ON). To prepare this solution, solid NADPH was dissolved in the reaction buffer to a final concentration of 18 mM. This solution was mixed using a vortex. NADPH solutions were protected from light at all times.

c) 7-methoxy-4-trifluoromethylcoumarin (MFC) – Substrate: A working solution of 25 mM MFC was made by dissolving solid MFC (BD Biosciences, Mississauga) into filtered acetonitrile (CH_3CN) in a 1.5 mL microtube. The working solution was vortexed and sonicated to ensure optimal mixing.

d) Sulfaphenazole – Positive control for CYP2C9 inhibition: A 50 mM stock solution of sulfaphenazole (Sigma-Aldrich Canada Inc, Oakville, ON, Cat #S0758) was made by dissolving pure compound into HPLC-grade MeOH. 40 µL of the stock solution was dissolved into 30 µL of pure MeOH, followed by 930 µL of distilled water. This yielded a 2 mM working solution in 7% MeOH.

e) Reaction Solution 1, 2, 3: Three reaction solutions were made according to Tables 3. 2- 3.4 below. These values have been optimized for one single well in the assay. If multiple wells were screened for fluorescence, the values were multiplied by the number of wells tested in the assay. Reaction Solution 1 contained the substrate (MFC) along with the catalyst (NADPH). Reaction Solution 2 contained denatured enzyme while Reaction Solution 3 contained the active enzyme. Reaction Solution 2 served as a control for background fluorescence.

Table 3. 2. Composition of reaction solutions 1, 2, and 3 for the CYP2C9*1 assays.

Reagent	**Reaction Solution 1 (µL)**	**Reaction Solution 2 (µL)**	**Reaction Solution 3 (µL)**
Distilled water	28.4	75	75
Reaction buffer – 0.5 M	64	10	10
NADPH – 18 mM	6	-	-
MFC – 2 mM	1.6	-	-
Active CYP2C9*1 – 1 µM	-	-	5
Denatured CYP2C9*1 – 1 µM	-	5	-
Total	**100**	**90**	**90**

Table 3. 3. Composition of reaction solutions 1, 2, and 3 for the CYP2C9*2 assays.

Reagent	Reaction Solution 1 (µL)	Reaction Solution 2 (µL)	Reaction Solution 3 (µL)
Distilled water	28.4	77.5	77.5
Reaction buffer – 0.5 M	64	10	10
NADPH – 18 mM	6	-	-
MFC – 2 mM	1.6	-	-
Active CYP2C9*2 – 2 µM	-	-	2.5
Denatured CYP2C9*2 – 2 µM	-	2.5	-
Total	**100**	**90**	**90**

Table 3. 4. Composition of reaction solutions 1, 2, and 3 for the CYP2C9*3 assays.

Reagent	Reaction Solution 1 (µL)	Reaction Solution 2 (µL)	Reaction Solution 3 (µL)
Distilled water	28.4	77.5	77.5
Reaction buffer – 0.5 M	64	10	10
NADPH – 18 mM	6	-	-
MFC – 2 mM	1.6	-	-
Active CYP2C9*3 – 2 µM	-	-	2.5
Denatured CYP2C9*3 – 2 µM	-	2.5	-
Total	**100**	**90**	**90**

An Applied Biosensors Cytofluor 4000 Fluorescence Measurement System plate reader (MTX Lab Systems Inc, Virginia, USA) was used and adjusted to the following settings using the Cytofluor software: lamp on, filter at excitation 409/20, emission at 530/25, gain 80, mix time of 3 seconds, Costar 96 well plate, and total incubation time of 20 minutes with 3 cycles.

Reaction solutions 1, 2, and 3 were first prepared with water and buffer. The solutions were kept at room temperature. The assay plate was then prepared using a vehicle control (7% MeOH), a positive control (sulfaphenazole in 7% MeOH), and test samples. For the vehicle and positive controls, 10 μL was directly aliquoted into the assay plate in triplicate. Based on a final in-well volume of 200 μL, the final concentration of vehicle and sulfaphenazole were 0.35% MeOH and 100 μM, respectively.

Similar to the CYP3A4 assays, it was necessary to ensure a low methanol concentration in all wells. 9 μL of distilled water was first plated, followed by the addition of 1 μL of extract. The final in-well concentration of all extracts tested was 80 mg/mL. Once the assay plate was prepared, it was kept in darkness.

Using the values listed in Tables 3. 2- 3. 4, NADPH and MFC were vortexed and added to Reaction Solution 1. After mixing the solution thoroughly, 100 μL was aliquoted into all reaction wells. The final in-well concentration of NADPH and MFC were 0.54 mM and 0.2 mM, respectively. Reaction Solution 2 was then prepared by adding CYP2C9 enzymes (BD Gentest, Mississauga, ON) previously denatured using a boiling water bath for 15 minutes. 90 μL of Reaction Solution 2 was aliquoted into the first set of vehicle, positive control, and test wells. Lastly, Reaction Solution 3 was made by adding active CYP2C9 enzymes (BD Gentest, Mississauga, ON). 90 μL of Reaction Solution 3 was aliquoted into the second set of vehicle, positive control, and test wells. The final in-well concentration of denatured or active enzyme was 25 nM in 0.185 M buffer. The assay plate was subsequently placed into the plate reader and scanned for fluorescence.

3.2.4 Quantification of Enzyme Activity

For each assay, triplicate values were obtained for each tested sample. The assays were replicated three times (n=3) to yield a total of 9 experimental values for each sample. Activity, expressed as fluorescence counts, was determined using equation [1]:

$$[1] \quad \text{Activity} = \frac{[(\text{Test } t=x) - (\text{Test } t=0)] - [(\text{Test blank } t=x) - (\text{Test blank } t=0)]}{[(\text{Vehicle } t=x) - (\text{Vehicle } t=0)] - (\text{Vehicle blank } t=x) - (\text{Vehicle blank } t=0)]}$$

where x represents the time point of interest and 'blank' represents the samples with denatured enzyme.

Percent inhibition was calculated using equation [2]:

$$[2] \quad \% \text{ Inhibition} = [(1-\text{Activity}) \times 100]$$

3.2.5 Determination of IC_{50} Values

The IC_{50} value of each ginseng product was determined by nonlinear regression analysis of the enzyme activity-concentration data using GraphPad Prism software (v5.0, GraphPad Software Inc, California, USA). The log-transformed data was fitted by equation [3]:

$$[3] \quad y = \text{Bottom} + (\text{Top} - \text{Bottom})/ ((1+10)^{x-\text{LogIC50}})$$

where x represents the concentration and y represents percent activity. The top and bottom parameters of the model were constrained to 100 and 0 percent activity, respectively.

3.2.6 HPLC Analysis of ginsenoside content

The ginseng products were analyzed using High Performance Liquid Chromatography with diode array detection (HPLC-DAD) to characterize them for six major ginsenosides (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd). Briefly, a Phenomenex Luna C18 column (150mm x 4.6mm; 5μm particle size; 100A pore size) was kept at 25°C and a flow rate of 1.5mL/min maintained. All solvents were HPLC-grade (Fisher Scientific, Ottawa, ON). The mobile phase consisted of water (A), 80:20 acetonitrile: water (B), and acetonitrile (C). Initial conditions of 76% A: 24% B were maintained for 8 minutes then changed following linear gradients to 68% A: 32% B in 18 minutes, 60% A: 40% B in 25 minutes, and 52% A: 48%B in 42 minutes. The column was washed with 100% C for the next 11 minutes and returned to initial conditions for a total run time of 53 minutes. 10 μL of sample was injected and the profile was monitored at 203 nm.

3.2.7 Statistical Analysis

All data were expressed as means ± standard mean of error (SEM). All statistical analysis was conducted using Graph Pad Statistical Software. For the CYP3A4 assay, the difference between the means of ginseng products was assessed by a 5 x 6 two-way analysis of variance (ANOVA) where six products were compared across 5 concentrations. For CYP2C9 assays, one-way ANOVAs were performed to compare the means across different ginseng products. Post-hoc, pair-wise comparisons were then computed with Bonferroni correction for all ginseng products. Linear regression analysis was used to assess the

relationship between ginsenoside content and inhibition activity. The significance level was set *a priori* at $p<0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Ginsenoside profiles

As shown in Table 3. 5, the total ginsenosides content in Ontario ginseng extracts were greater than the other tested extracts. In particular, the total ginsenosides in Ontario ginseng extracts ranged from 10.7-11.4% when compared to the other products, which ranged from 4.18-5.09%. Composition was fairly consistent between Ontario samples, which differed from the composition in the Asian ginseng sample. Our results showed that Rb1 was the most abundant ginsenoside in all quantified *Panax quinquefolius* ginseng products while Rb2 was the least abundant ginsenoside (Table 5). Furthermore, with the exception of *Panax ginseng* and Ontario ginseng (MeOH), Re was found to be the second most abundant ginsenoside across products. Ginsenoside profiles could not be established for Red Chinese Ginseng and Korean Ginseng due to the existence of excipients interfering with the HPLC-DAD detection. Although these samples could not be quantified, marker compounds indicative of *Panax ginseng* were identified.

Table 3. 5. Characterization of ginsenoside content and composition across products through HPLC-DAD analysis.

Ginseng Product[a]	***Ginsenoside Profile***						
	Rg1 (%)	**Re (%)**	**Rb1 (%)**	**Rc (%)**	**Rb2 (%)**	**Rd (%)**	**Total (%)**
NPN3	0.160	1.04	2.33	0.258	0.059	0.324	**4.18**
NPN4	0.201	0.857	2.30	0.670	0.120	0.712	**4.86**
Panax Ginseng (MeOH)	1.24	0.355	1.73	1.00	0.576	0.184	**5.09**
Ontario Ginseng (EtOH)	0.509	3.26	5.16	0.840	0.187	0.793	**10.7**
Ontario Ginseng (Aqueous)	0.542	3.37	5.79	0.803	0.199	0.687	**11.4**
Ontario Ginseng (MeOH)	0.160	1.86	5.92	0.814	0.156	2.24	**11.1**

[a] Only products with chromatograms suitable for quantification were presented. Due to the presence of excipients, NPN1 and NPN2 could not be quantified using HPLC-DAD. These products were omitted in subsequent statistical comparisons and correlations.

3.3.2 Concentration-dependent inhibition of CYP3A4 by ginseng extracts

Our results showed that all ginseng products had weak to moderate inhibitory activity, depending on the CYP enzyme studied. For CYP3A4, IC_{50} values ranged from 35.15 μg/mL to 248.2 μg/mL. Ontario ginseng roots were significantly more inhibitory than any other tested product (Figure 3. 1). The degree of CYP3A4 inhibition by different ginseng products was correlated with total ginsenoside content (% w/w). The correlation between inhibitory activity and ginsenoside content was statistically significant with an R^2 value of 0.99 (Figure 3. 2). Such interactions could cause the plasma levels of concomitantly-administered drugs to be higher, posing a potential risk for serious herb-drug interactions. While the *in vitro* results predict a possible interaction, further clinical studies are needed to determine whether the effect can occur *in vivo*.

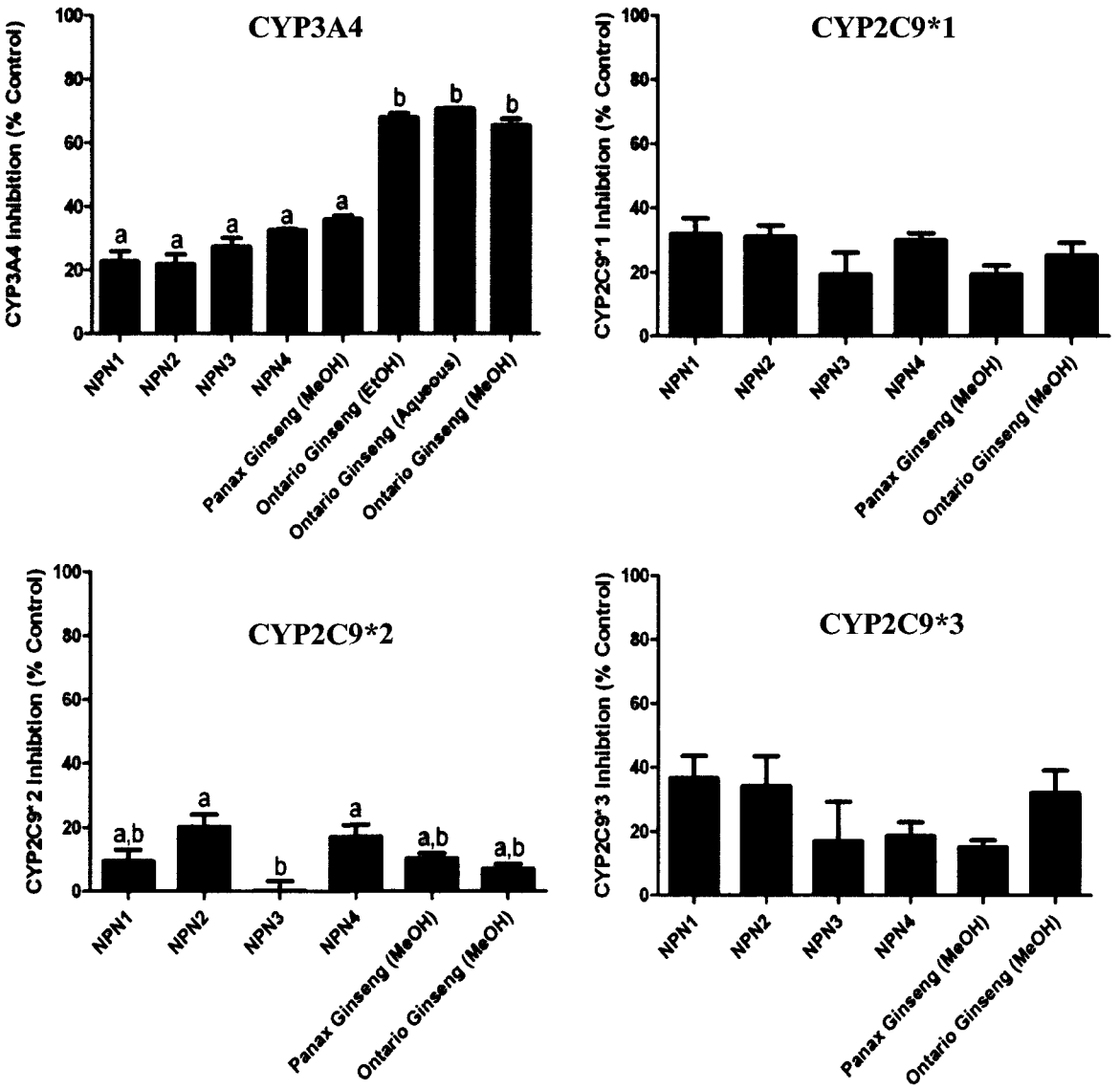

Figure 3. 1. The inhibitory potential of different ginseng products on CYP3A4 and three CYP2C9 allelic variants. A typical 200 μL reaction mixture contained cDNA-expressed enzymes (0.3 pmol for CYP3A4, 5 pmol for CYP2C9*1, 5 pmol for CYP2C9*2, and 10 pmol for CYP2C9*3), NADPH (0.54 mM), probe substrates (1 μM dibenzylfluorescein for CYP3A4, 0.2 mM MFC for CYP2C9 assays), and ginseng samples (80 μg/mL) in 185 mM reaction buffer. Except for CYP2C9*3 whose incubation time was set to 60 minutes, all other CYP assays were incubated for 20 min at 37°C. All data were expressed as means ± SEM. Samples were tested in triplicate with assays repeated three times (n=3). One-way ANOVA was performed, followed by Bonferroni multiple comparison tests. Means with significant differences are distinguished by different letters ($p<0.05$).

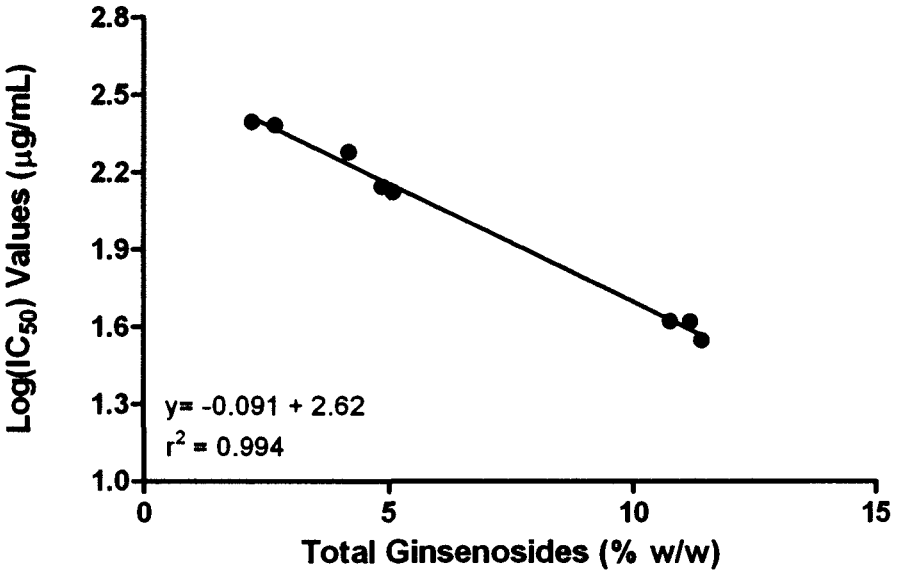

Figure 3. 2. Correlation between total ginsenoside content and log-transformed IC_{50} values. The IC_{50} values of different ginseng products were plotted against their total ginsenoside content. Only products quantified by HPLC-DAD were included in the analysis. Pearson correlation yielded a value of r = -0.997 (p<0.0001).

3.3.3 Inhibition of CYP2C9 allelic variants by ginseng extracts

When the three allelic variants of CYP2C9 were studied, all ginseng products had relatively weak inhibitory activity on the wild-type allelic variant, CYP2C9*1, when compared to control activity and inhibition was lower than inhibition of CYP3A4 (Figure 3.1). Inhibitory activity ranged from 19.2% to 31.8% of the control. The CYP2C9*3 variant gave a similar profile of inhibition to the wild-type enzyme (15.1% to 36.6%), but there was low inhibition of CYP2C9*2 by all products (0.1% to 20.1%). No significant correlation was found between ginsenoside content and inhibition of any of the three CYP2C9 variants. These results demonstrated that genetic mutations did not lead to differences in the inhibition of CYP2C9 or its allelic variants. This suggests that genetic background may not be involved in the predisposition of herb-drug interactions for ginseng with this enzyme.

3.4 CONCLUSIONS AND FUTURE DIRECTIONS

Herb-drug interactions are of growing concern due to the increased use and awareness of natural health products. They generally arise when natural health products inhibit CYP enzymes, altering the rate of metabolism for other drugs in the system. It is important to note, however, that interactions may also arise when CYP enzymes are induced, resulting in reduced plasma drug levels. Alternatively, CYP enzymes could also undergo mechanism-based inhibition, whereby a CYP enzyme can be completely inactivated by covalent bonding to a component of the herb. Furthermore, botanicals can elicit a biphasic cellular response, whereby CYP activity may be inhibited initially, followed by induction after prolonged incubation or repeated administration. Such factors would need to be

considered in future studies in order to establish the true risk of ginseng in herb-drug interactions.

Although the results from the present study suggest that the risk of herb-drug interactions is low even in the presence of polymorphisms, extrapolating *in vitro* findings to clinical settings is difficult due to a number of extraneous factors that need to be considered. For example, our study only investigated the effect of ginseng products on human cDNA-expressed enzymes with a single substrate. In clinical practice, different CYP2C9 genotypes including CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*3 with varying amounts of probe compounds would need to be considered before drawing conclusions. Furthermore, these genotypes only represent a limited subset of all possible combinations, and are associated with different pharmacokinetic profiles. Such complex relationships have been a common challenge in the field of pharmacogenetics and further research would be required to establish the true clinical relevance of polymorphisms in herb-drug interactions.

The risk of developing herb-drug interactions is affected by many factors, including the quality and species of the herb used, the concentration of active CYP enzyme inhibitors, as well as the intrinsic and extrinsic factors that affect drug disposition in an individual. The significance of the particular drug-metabolizing enzyme studied and ginsenoside profile is apparent. It is important for future research to begin examining the impact of genetic polymorphisms on drug metabolism and how such differences could lead to herb-drug interactions as well as examining metabolite profiles of herbs in relation to CYP activity. The

results of such studies will ultimately help predict and prevent serious herb-drug interactions for natural health product users.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

PREFACE

This study was initiated to characterize Ontario ginseng beyond the six major ginsenosides which were studied in Chapter 2 and which are the most commonly studied compounds in ginseng in the published literature. The LC/MS/MS method for ginsenoside analysis was developed by Ammar Saleem and myself and was developed using a method from Anthony Windust (NRC, Ottawa, Ontario) as a starting point. The HPLC-ELSD method for monosaccharide analysis was also developed by myself and Ammar Saleem. I conducted the method validation and carried out the chromatography experiments.

4.0 ADVANCED PHYTOCHEMICAL CHARACTERIZATION OF ONTARIO GINSENG (*PANAX QUINQUEFOLIUS*)

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ABSTRACT

Ginsenosides are the most commonly studied compounds in ginseng and typically, five or six major ginsenosides are assessed. This study used an LC/MS/MS method to examine a wider range of ginsenosides in Ontario ginseng including Rg1, Re, Rb1, Rb2, Rc, Rd, Ro, and malonyl ginsenosides mRb1, mRb2, mRc, and mRd. Neutral ginsenoside composition generally followed the trend: Rb1> Rd≈ Re> Rc> Ro> Rg1> Rb2~ Rg1. Of the malonyl ginsenosides, mRb1 was present in the highest quantities and together malonyl ginsenosides accounted for up to 16.45% of total ginsenoside content. This showed that malonyl ginsenosides can make up a significant percentage of total ginsenoside content and should be included in ginsenoside quantification. Ginsenoside polysaccharides have been shown to have immunomodulatory activity, however polysaccharides have not been well characterized in *P. quinquefolius*. As a first step in polysaccharide identification, the monosaccharide composition of Ontario ginseng was assessed using a newly developed HPLC-ELSD method. Glucose was the major monosaccharide identified in Ontario ginseng root samples. Galacturonic acid, galactose, and arabinose were also detected in significant quantities.

4.1 INTRODUCTION

Ginsenosides are the most commonly studied compounds in ginseng as they are commonly believed to be responsible for most of ginseng's biological activity. Although greater than 150 ginsenosides have been identified (Christensen, 2008), most studies examine only 5 or 6 of the major ginsenosides by chromatographic methods. The majority of HPLC methods developed to study ginsenosides in North American ginseng, *Panax quinquefolius*, examine the major ginsenosides Rg1, Re, Rb1, Rb2, Rc, and Rd. These

methods tend to be long, ranging 45- 118 minutes and often do not examine common malonyl ginsenosides or oleanic type ginsenoside, Ro.

Common malonyl ginsenosides include malonyl mRb1, mRb2, mRc, and mRd. These correspond to their protopanaxadiol ginsenoside counterparts but have a malonyl group attached to the glucose at the R_1 group (Figure 4. 1a). Malonyl ginsenosides can contribute to a significant proportion of total ginsenoside levels in American ginseng and ignoring them can lead to underestimating ginsenoside levels (Awang, 2000). Furthermore, as malonyl ginsenosides are converted to neutral ginsenosides under gastric conditions (Awang, 2000), failure to include malonyl ginsenosides in analysis would lead to underestimating the amount and composition of individual ginsenosides that would effectively be taken up by the body. Due to these reasons, it is useful to know the content of malonyl ginsenosides in root samples as this contributes to total ginsenoside content. Along with malonyl ginsenosides, oleanic type ginsenoside Ro (Figure 4. 1b) is rarely included in analysis often due to low availability of this compound as a pure standard. This ginsenoside may also contribute significantly to overall ginsenoside levels or contribute to the unique biological effects of ginseng.

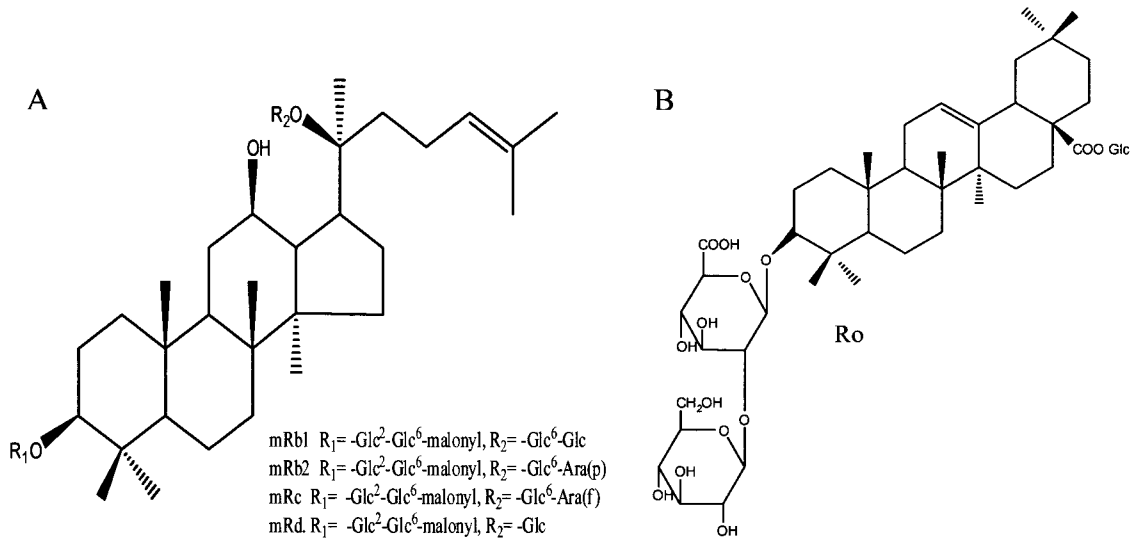


Figure 4. 1. Structures of malonyl ginsenosides (A) and ginsenoside Ro (B).

Although ginsenosides are thought to be responsible for ginseng's therapeutic effects, other compounds have also been shown to have significant biological activity. Specifically, the polysaccharide fraction of *P. quinquefolius* has been shown to have immunomodulatory effects *in vitro* and in clinical studies (Assinewe et al., 2002;Biondo et al., 2008;Predy et al., 2006). The most valuable ginseng product is CV Technologies Cold FX, a clinically tested American ginseng polysaccharide derivative which has an estimated $48 million dollar market. Polysaccharides have not been well characterized in *P. quinquefolius*. The first step in identifying polysaccharide compounds is to examine the contributing monosaccharide components. Several polysaccharides have been identified in *P. ginseng* and *P. notoginseng* but these compounds, including arabinogalactan, pectins, and acidic polysaccharides, have been rarely studied in the North American species, *P. quinquefolius*.

In this study, an LC/MS/MS method was developed for ginsenoside analysis, including the major ginsenosides Rg1, Re, Rb1, Rb2, Rc, and Rd, as well as the oleanic-type ginsenoside Ro and malonyl ginsenosides mRb1, mRb2, mRc, and mRd. Monosaccharide components of Ontario *P. quinquefolius* roots were also examined using a newly developed HPLC-ELSD method. This study expands the characterization of Ontario ginseng roots and introduces new analytical methods for the study of ginsenosides and monosaccharide components.

4.2 MATERIALS AND METHODS

4.2.1 Materials and Sample Preparation

For ginsenoside analysis, ginseng roots were assessed from two farms in Norfolk County in Southern Ontario. Five roots were examined from each farm. Roots were extracted 3 times by sonication in 70% methanol as described by McIntyre et al. (McIntyre et al., 2010, in press). Sonication was performed at room temperature as malonyl ginsenosides are susceptible to thermal degradation (Court et al., 1996). For each sample, two aliquots were prepared. One aliquot was analyzed as the unmodified 70% methanol extract (M) and the other aliquot was hydrolyzed (H) before analysis. This was done in order to quantify malonyl ginsenoside components, as malonyl ginsenosides are converted to their neutral counterparts upon hydrolysis (Court et al., 1996). For hydrolysis, 100 µL of 5% potassium hydroxide (KOH) was added to 1mL of extract and incubated at room temperature in darkness for 2 hours. To neutralize the extract, 100 µL of 14% KH_2PO_4 was added after incubation. Samples were diluted 10 times for LC/MS/MS analysis. For both the unmodified (M) and hydrolyzed (H) extracts, samples were filtered through a 0.2 µm PTFE filter (Chromatographic Specialties Inc., Brockville, ON).

For analysis of monosaccharide components, five ginseng roots were obtained from one farm in Norfolk County, Ontario. Ground ginseng root was mixed with water in a polypropylene tube and extracted in a 90°C water bath for 2 hours, cooled to room temperature and centrifuged for 5 minutes. The supernatant was then transferred to a second tube. Polysaccharides were precipitated by adding 95% ethanol to the supernatant in a 4:1 ratio (ethanol: supernatant). The tube was then mixed using a vortex and placed in the refrigerator for 10 minutes. The sample was then centrifuged and the supernatant removed and discarded. The polysaccharide pellet was dried, accurately weighed, and re-suspended in 2mL of 6N HCl to hydrolyze the polysaccharides into their monosaccharide components. The suspension was heated in a 90 °C water bath for 2 hours and then cooled to room temperature. The pH was then adjusted to approximately 7 with NH_4OH and centrifuged. The supernatant was aspirated into a second tube and filtered using a 0.2 μm nylon filter (Chromatographic Specialties Inc., Brockville, ON).

4.2.2 LC/MS/MS Ginsenoside analysis

LC/MS/MS analysis was conducted using a 3200 QTRAP (Applied Biosystems by Life Technologies, Carlsbad, California) consisting of a binary pump, autosampler, and turbo spray ion source. A Waters Symmetry Shield RP8 column (3.5 μm, 2.1 x 50mm) was kept at 40 °C and a flow rate of 0.4 mL/min was maintained. Solvents were Chromasolv LC-MS grade (Sigma Aldrich, Oakville, Ontario) and consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid. Initial LC conditions were 80:20 A:B which was changed linearly over 20 minutes to 70:30 A:B. The column was washed with 10:90 A:B for 3 minute before returning to initial conditions over the next 2 minutes for a total run time of 25

minutes. Between each sample, conditions were allowed to equilibrate for 5 minutes. Samples were tested in triplicate and 2 μL of sample was injected.

Multiple reaction monitoring (MRM) and enhanced mass scan (EMS) experiments were performed. MRM ions were selected by directly injecting pure standards and scanning product ions to determine the most abundant ions for MRM. The parameters that were used in the MRM scan are presented in Table 4. 1. Ginsenosides sharing the same fragmentation pattern were differentiated from one another based on retention time in comparison to standards. The scan was conducted in positive mode with a cycle of 0.9546ms and 1542 cycles in the 25 minute method.

The EMS experiment was used to monitor ions of malonyl ginsenosides. For malonyl ginsenosides mRd, mRc, and mRb2, molecular weight + H and molecular weight + H + Na adduct were selected as the ions to scan. A width of 5 amu on either side was also scanned. For mRb1, the ion mass 1132 amu was monitored as this was determined to be the most abundant ion by direct injection and fragmentation of a pure mRb1 standard (isolated at Dr. Anthony Windust's lab, NRC, Ottawa, Ontario).

Table 4. 1. Ion masses scanned in quadrupole 1 (Q1) and quadrupole 2 (Q2) in the MRM experiment corresponding to individual ginsenosides.

Ginsenoside	Q1 Mass (amu)	Q2 Mass (amu)	Time (msec)
Rg1	807.344	365.2	50
Re/ Rd	969.352	789.4	50
Ro	979.308	641.3	50
Rc/ Rb2	1101.367	335.2	50
Rb1	1131.409	365.1	50

Calibration curves were created using pure standards of Rg1, Re, Rb1, Rc, Rb2, Rd (Indofine Chemical Company Inc., Hillsborough, New Jersey), and Ro (isolated at Dr. Anthony Windust's lab, NRC, Ottawa, Ontario) by injecting a standard mix at 6 injection volumes. Inter- and intra-day variation was examined using these pure standards. Quantification was done using Analyst Software (Applied Biosystems by Life Technologies, Carlsbad, California) and samples were quantified based on the calibration curve with the greatest calculated accuracy. Malonyl ginsenosides were quantified by subtracting the calculated amount of their neutral ginsenoside counterpart in the unmodified extract from the amount calculated in the sample after hydrolysis.

4.2.3 HPLC-ELSD Monosaccharide analysis

HPLC analysis was conducted using an Aglient system consisting of an autosampler, quaternary pump, and evaporative light scattering detector (ELSD) (Agilent Technologies, Mississauga, Ontario). Due to co-elution of monosaccharides, analysis was performed using two separate columns. To examine glucose, galactose, and arabinose, a Rezex RPM

Monosaccharide PB+2 (8%) (Phenomenex, Torrance, California) column was used. Mannose and xylose were also separated using this column but were not detected in any ginseng samples. The column was kept at 80 °C and a flow rate of 0.6 mL/min was maintained. Solvent conditions were held isocratically at 100% water (Chromasolv Plus, HPLC- grade, Sigma Aldrich, Oakville, Ontario) for 25 minutes. The ELSD temperature was set to 80 °C and parameters were set as follows: gain 5; offset 1; sampling time 100-10Hz; filter 5s. Samples were tested in triplicate and 20 μL of sample was injected.

Galacturonic acid and rhamnose content were examined using a Luna 5 μ NH2 100Å column (Phenomenex, Torrance, California). The column was kept at 40 °C and a flow rate of 3 mL/min was maintained. The solvents were Chromasolv Plus HPLC-grade (Sigma Aldrich, Oakville, Ontario) and consisted of acetonitrile (A) and water (B). Conditions were held isocratically at 80:20 A:B for the total run time of 5 minutes. The ELSD temperature was set to 44 °C and parameters were set as follows: gain 1, offset 0, sampling time 100-10Hz, filter 5s. Samples were tested in triplicate and 20 μL of sample was injected.

Quantification was based on the linear portion of calibration curves of pure standards injected at 9 concentrations. Inter- and intra-day variation was examined using pure standard compounds. Quantification was carried out using Chem Station Software (Agilent Technologies, Mississauga, Ontario) and was based on the calibration curves with the greatest accuracy.

4.2.4 Statistical analysis

Statistical analysis was conducted using S-Plus 8.0 software. To assess the difference in ginsenoside content between farms, an ANOVA test with a Tukey's post-hoc test. Results were considered significant when $p<0.05$.

4.3 RESULTS AND DISCUSSION

5.3.1 LC/MS/MS method validation

Calibration curves were produced for standard compounds (Rb1, Rg1, Rd, Ro, Rb2, Re, Rc, and Rb1) and showed quadratic response profiles ($R^2=0.99$) for all quantified compounds when injection volumes of 4, 3, 2, 1, and 0.1 µL were included. An injection volume of 5 µL was also assessed but determined to be outside of the quadratic response range.

To assess the precision and accuracy of the method, variation in the calculated concentration of standard compounds was assessed within and between days of analysis. Each day, each level was injected three times and variation is expressed as the percent coefficient of variation (%CV= standard deviation/mean amount x 100). Table 4. 2 shows intra- and interday variation. Variation was similar between days, being slightly reduced on day 3. Interday variation was generally under 10%, with the exception of interday variation of standards injected at 0.1 µL which ranged from 7.26- 28.16% variation. Limit of detection was recorded at a peak height of 60 cps and limit of quantification at 180 cps.

Table 4. 2. Intra- and inter-day variation of ginsenosides detected by LC/MS/MS.

Ginsenoside	injection volume (μL)	Intraday variation (%CV)			Interday variation
		Day 1	Day 2	Day 3	
Rg1	4	5.66	7.73	19.27	6.34
	3	21.88	3.81	0.70	13.64
	2	4.38	17.64	8.65	8.87
	1	12.50	4.81	5.71	3.79
	0.1	8.58	3.89	3.88	11.30
Rd	4	5.15	2.49	5.08	2.06
	3	0.53	2.68	0.97	2.86
	2	0.50	1.47	1.65	1.19
	1	1.06	3.07	0.86	2.93
	0.1	3.48	3.63	1.11	10.14
Ro	4	5.23	2.15	1.39	3.09
	3	4.08	3.88	2.33	4.12
	2	4.74	4.70	1.49	1.69
	1	1.42	0.70	1.63	1.97
	0.1	2.67	10.68	4.75	7.26
Rb2	4	4.10	2.56	4.34	0.81
	3	1.38	1.84	0.97	1.65
	2	2.99	1.44	0.51	0.93
	1	0.58	2.63	2.68	4.66
	0.1	2.37	2.04	0.32	13.18
Rb1	4	2.72		1.58	3.41
	3	0.99	0.49	2.95	3.50
	2	1.25	1.82	1.23	1.51
	1	1.56	1.74	2.39	6.48
	0.1	1.94	2.04	0.15	18.62
Re	4	6.09	2.83	2.22	0.69
	3	2.97	2.80	1.77	1.57
	2	2.57	0.30	1.30	1.80
	1	3.16	4.47	3.85	5.65
	0.1	6.43	9.06	3.49	28.16
Rc	4	2.06	0.67	2.85	2.59
	3	0.88	3.33	0.89	1.08
	2	1.71	2.18	1.39	1.99
	1	4.63	5.19	0.89	1.45
	0.1	1.61	1.83	1.18	16.76

4.3.2 LC/MS/MS Ontario ginseng ginsenoside analysis

A representative MRM chromatogram is presented in Figure 4. 2. There was variation in the content of individual ginsenoside between root samples in both populations and variation was similar between unmodified (M) and hydrolyzed (H) extracts as expected (Table 4. 3). Neutral ginsenoside composition generally followed the trend: Rb1>Rd≈Re>Rc>Ro>Rg1>Rb2~Rg1. Ro is not commonly assessed in *P. quinquefolius* but was found to be present at levels up to 2.95 mg/g dry weight. Ginsenoside Ro has been shown to contribute to the biological activity of ginseng with demonstrated anti-inflammatory activity and anti-hepatic activity in *in vivo* rat models (Matsuda et al., 1991;Matsuda et al., 1990). Due to the low availability of Ro as a standard, its biological effects have not been extensively studied. As more effects of Ro are determined, there may be a market for roots with greater Ro content.

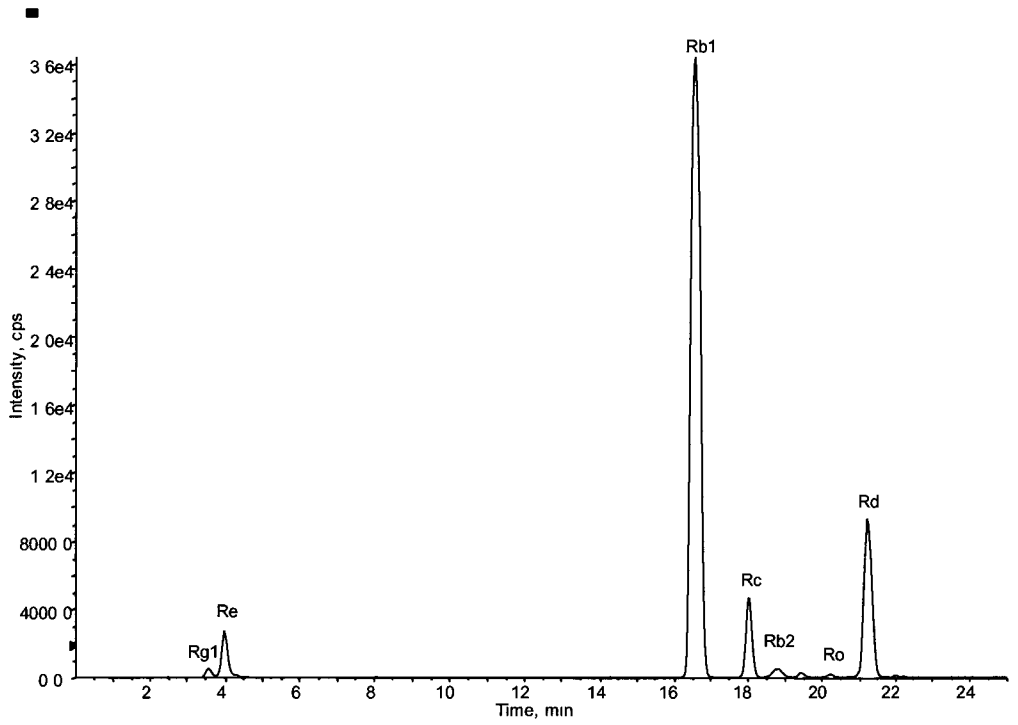


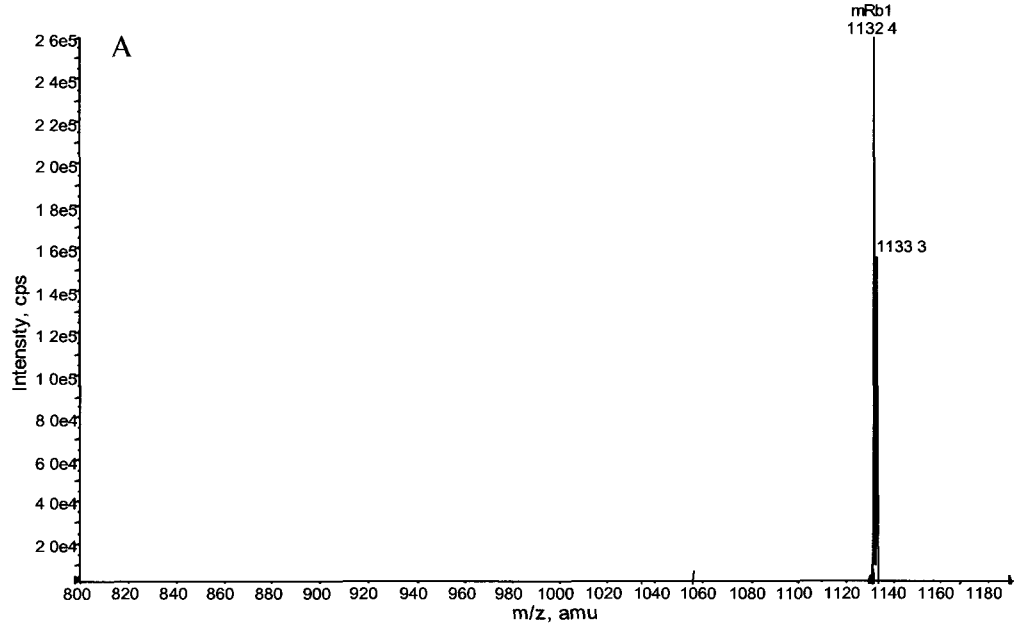
Figure 4. 2. Representative MRM chromatogram of one Ontario ginseng root.

Table 4. 3. Range of ginsenoside content (mg/g dry weight) in ginseng samples (n=5) grown at two Ontario ginseng farms before (M) and after hydrolysis (H).

	Range of Ginsenoside content (mg/g dry weight) (Mean (SEM))							
Samples	**Rg1***	**Rd**	**Ro**	**Rb2**	**Rb1**	**Re**	**Rc**	**Total**
Farm 1M	blq- 0.22 (0.12)	3.33 (0.05)- 9.22 (0.12)	0.57 (0.07)- 1.97 (0.08)	0.36 (0.01)- 0.43 (0.01)	22.47 (0.32)- 30.80 (0.36)	2.46 (0.71)- 4.05 (0.03)	1.86 (0.07)- 4.13 (0.09)	32.4 (0.40)- 49.14 (0.23)
Farm 2M	0.53 (0.05)- 2.40 (0.08)	1.91 (0.05)- 6.62 (0.40)	1.14 (0.02)- 2.95 (0.14)	0.30 (0.01)- 0.74 (0.02)	21.59 (0.13)- 36.61 (0.98)	4.19 (0.35)- 10.90 (0.11)	1.66 (0.21)- 10.68 (0.24)	32.22 (0.70)- 51.47 (0.16)
Farm 1H	blq- 0.12 (0.01)	3.46 (0.04)- 9.23 (0.25)	0.48 (0.05)- 2.34 (0.06)	0.37 (0.01)- 4.60 (0.01)	23.34 (0.22)- 32.90 (0.16)	2.69 (0.66)- 4.40 (0.18)	1.64 (0.11)- 3.46 (0.04)	33.32 (0.53)- 49.53 (0.62)
Farm 2H	0.45 (0.08)- 2.46 (0.11)	2.41 (0.12)- 7.09 (0.11)	0.97 (0.06)- 2.64 (0.11)	0.29 (0)- 0.59 (0.01)	22.14 (0.26)- 41.27 (0.37)	3.86 (0.19)- 11.22 (0.05)	1.96 (0.13)- 11.36 (0.13)	34.07 (0.30)- 58.01 (0.28)

*Ginsenoside content differed significantly between farms (ANOVA and Tukey's post-hoc test, $p<0.5$)

Monitoring ions representative of the malonyl ginsenosides, it was clear that ion abundance diminished significantly or was undetectable after hydrolysis (Figure 4. 3). Furthermore, the amount of the neutral ginsenoside counterparts increased following hydrolysis, indicating that malonyl ginsenoside are indeed present in Ontario ginseng. Malonyl ginsenosides were shown to occur in amounts from 1.14- 7.32 mg/g dry weight. The composition of malonyl ginsenosides observed in this study is in line with previous studies (Court et al., 1996;Christensen et al., 2006), showing the general trend for malonyl ginsenoside composition in *P. quinquefolius* to be: mRb1> mRd> mRc >mRb2 (Figure 4. 4). As shown in Table 4. 4, malonyl ginsenosides were responsible for up to 16.45% of the total ginsenoside content in a given root. As malonyl ginsenosides can make up a substantial percentage of total ginsenoside content, failure to including malonyl ginsenosides in analysis could cause total ginsenoside content as well as the content of the neutral ginsenoside counterparts to be significantly underestimated. As described by Awang (2000), when ingested malonyl ginsenosides are de-malonylated to neutral ginsenosides so that they can then be converted to deglycosylated saponins by intestinal bacteria. According to rat studies, only deglycosylated saponins have been detected in the plasma and intestinal tract (Awang, 2000). As malonyl ginsenosides are converted before being absorbed by the body, it is more accurate to include malonyl ginsenosides in total ginsenoside estimates. Variation in ginsenoside content has been shown to influence to some biological activities (Sievenpiper et al., 2003;Luu et al., 2010) so obtaining an accurate estimate of ginsenoside content is essential.


A
mRb1
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1133 3
Intensity, cps
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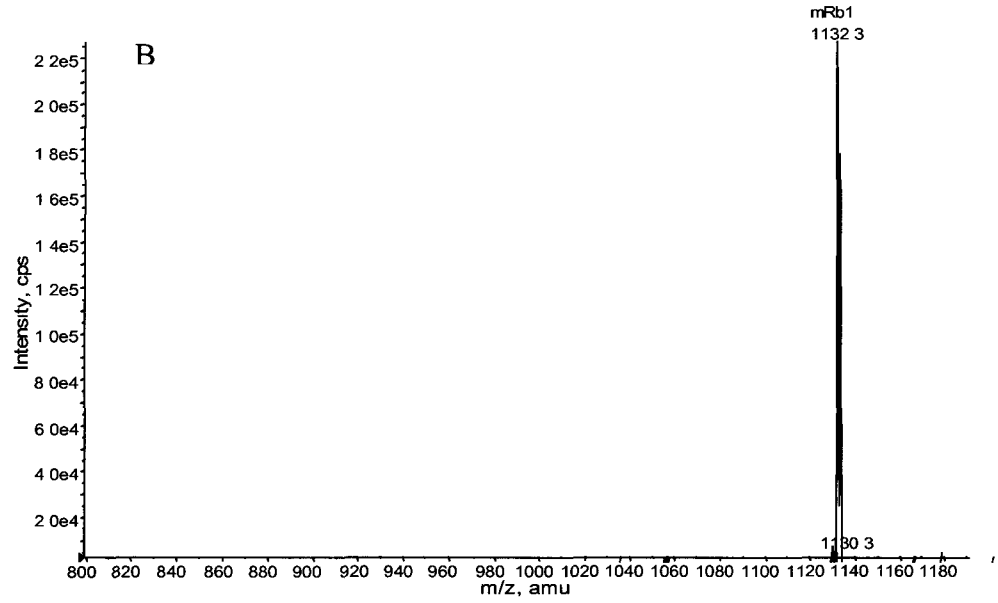
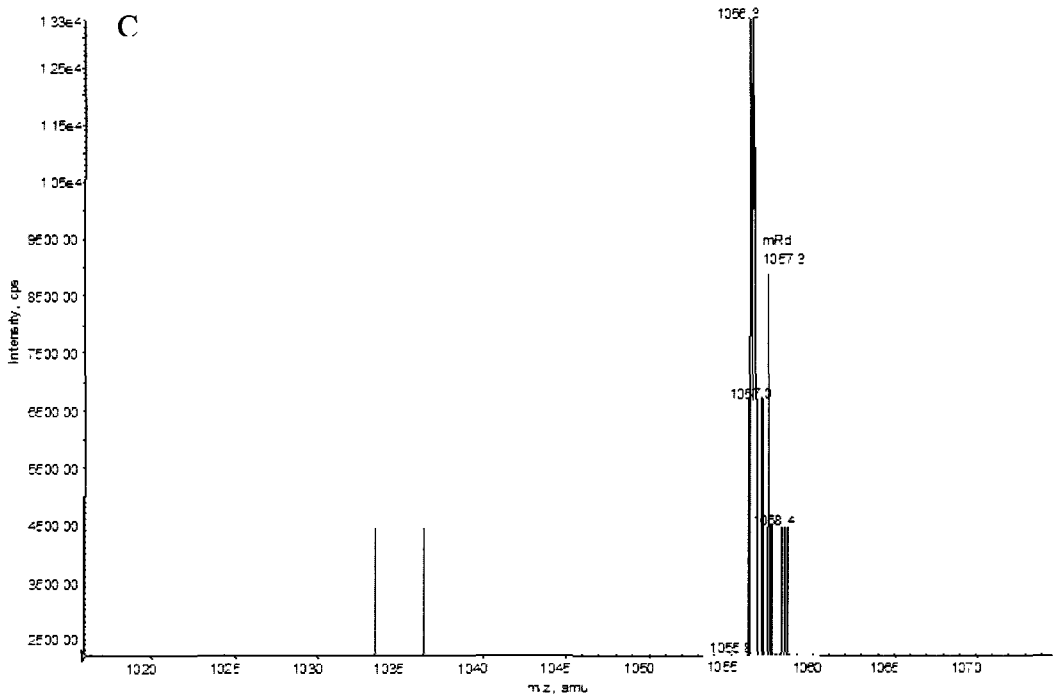
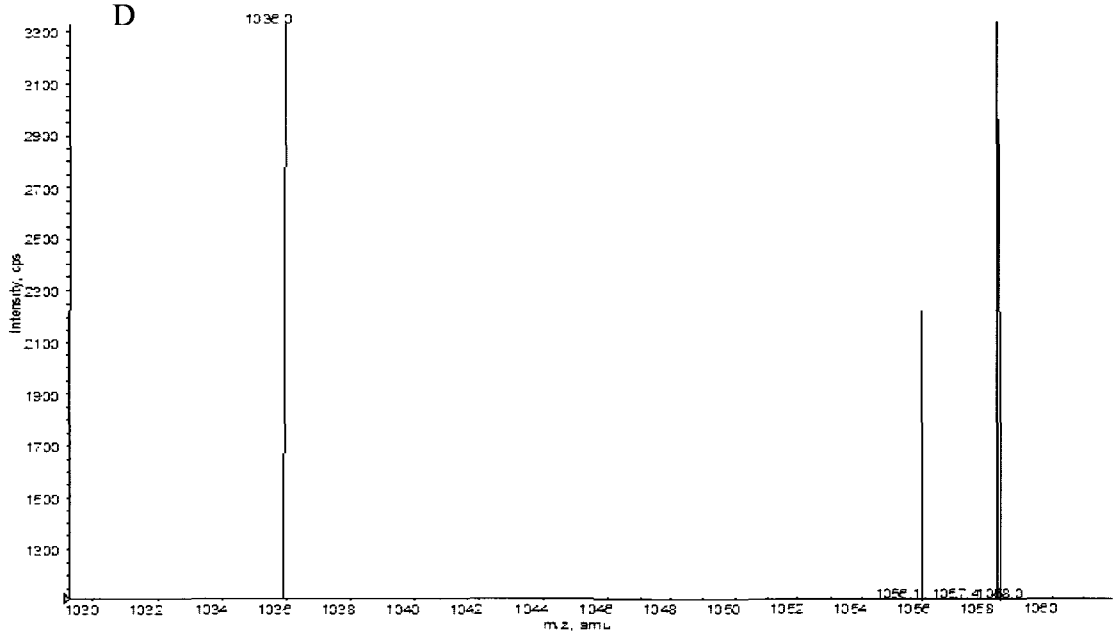
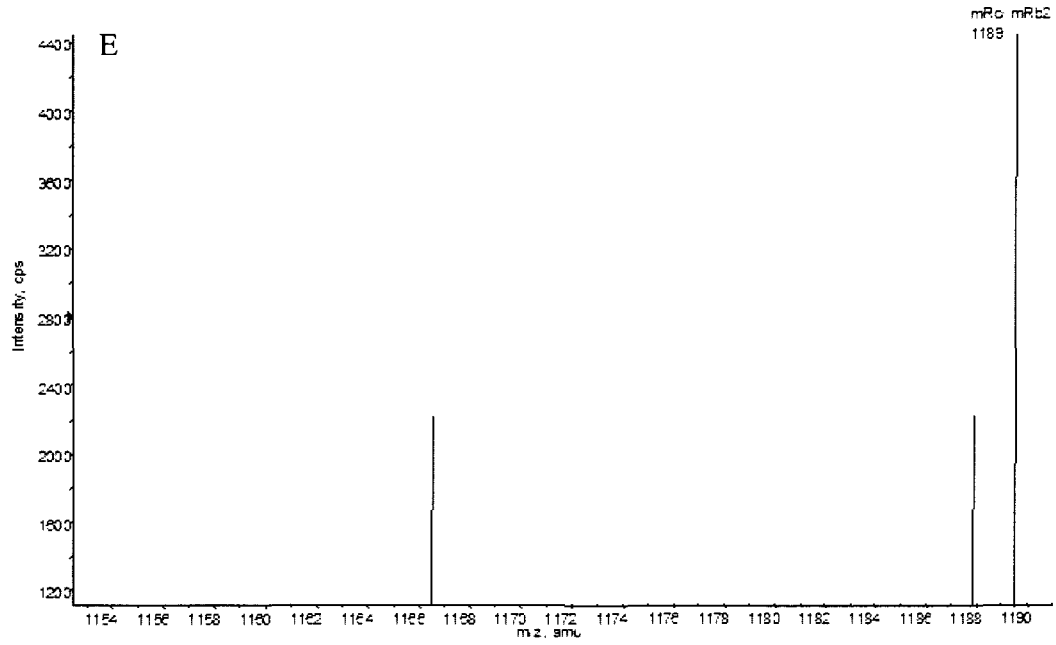
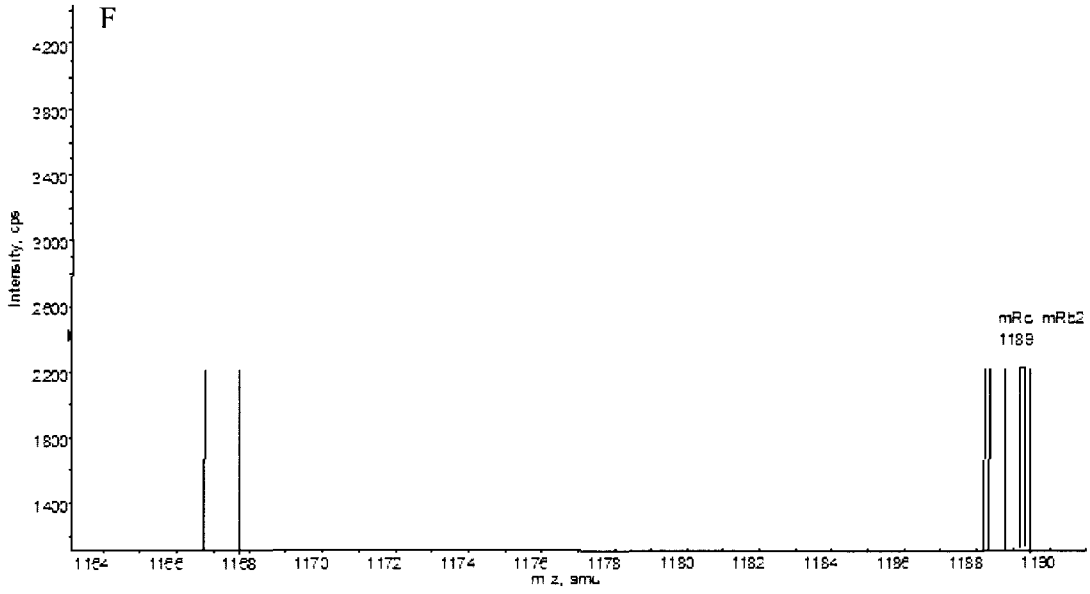
2 4e5
2 2e5
2 0e5
1 8e5
1 6e5
1 4e5
1 2e5
1 0e5
8 0e4
6 0e4
4 0e4
2 0e4
800 820 840 860 880 900 920 940 960 980 1000 1020 1040 1060 1080 1100 1120 1140 1160 1180
m/z, amu


B
mRb1
1132 3
1130 3
Intensity, cps
2 2e5
2 0e5
1 8e5
1 6e5
1 4e5
1 2e5
1 0e5
8 0e4
6 0e4
4 0e4
2 0e4
800 820 840 860 880 900 920 940 960 980 1000 1020 1040 1060 1080 1100 1120 1140 1160 1180
m/z, amu


C
Intensity, cps
m/z, amu
mRd
1057.9


D
Intensity, cps
m/z, amu


E
mRc mRb2
1189
Intensity, cps
m z, amu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mRc mRb2
1189
Intensity, cps
m z, amu

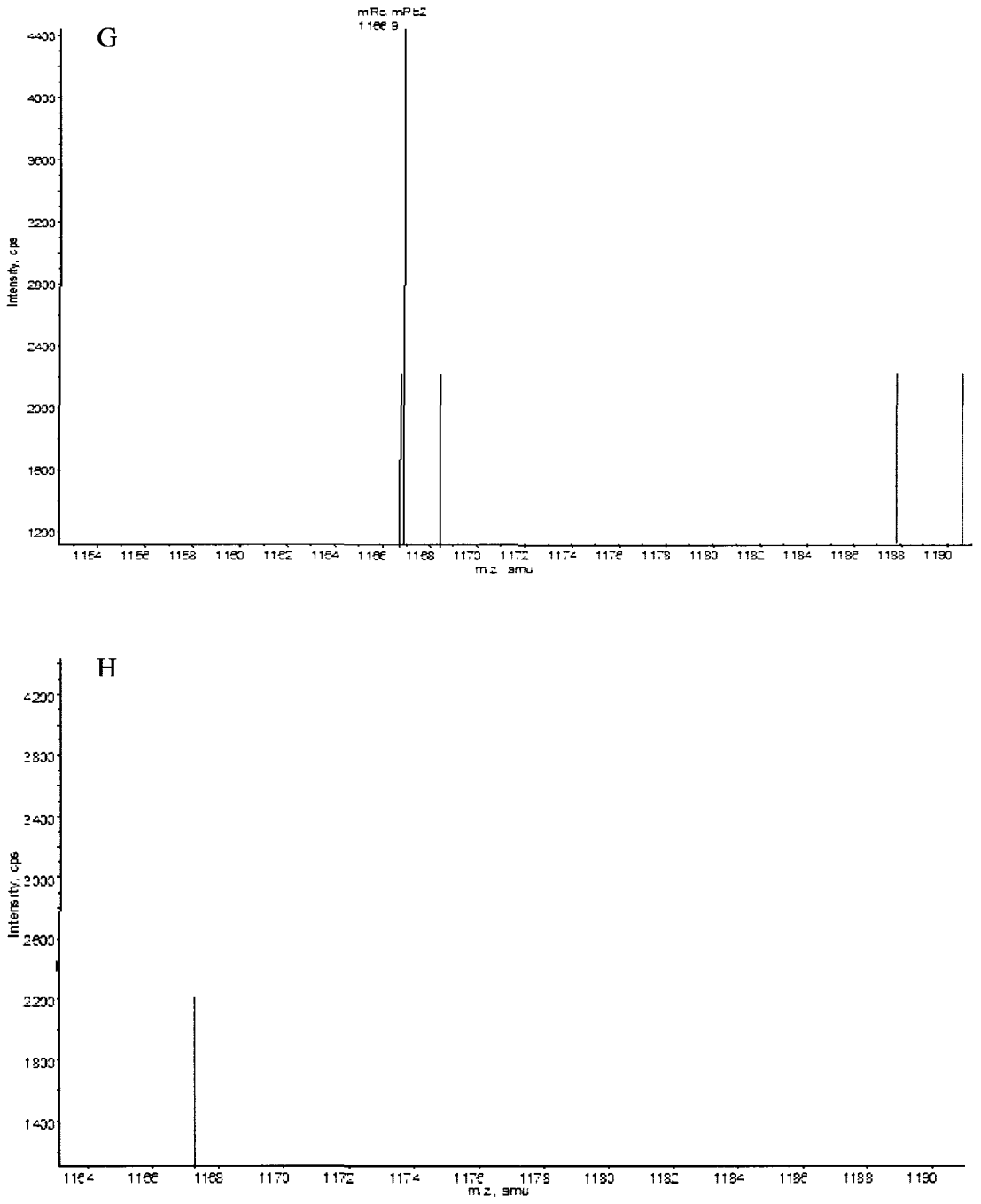

Figure 4. 3- EMS experiments showing mRb1 before hydrolysis (A), mRb1 after hydrolysis (B), mRd before hydrolysis (C), mRd after hydrolysis (D), mRc/mRb2 before hydrolysis (E and G), mRc/mRb2 after hydrolysis (F and H).

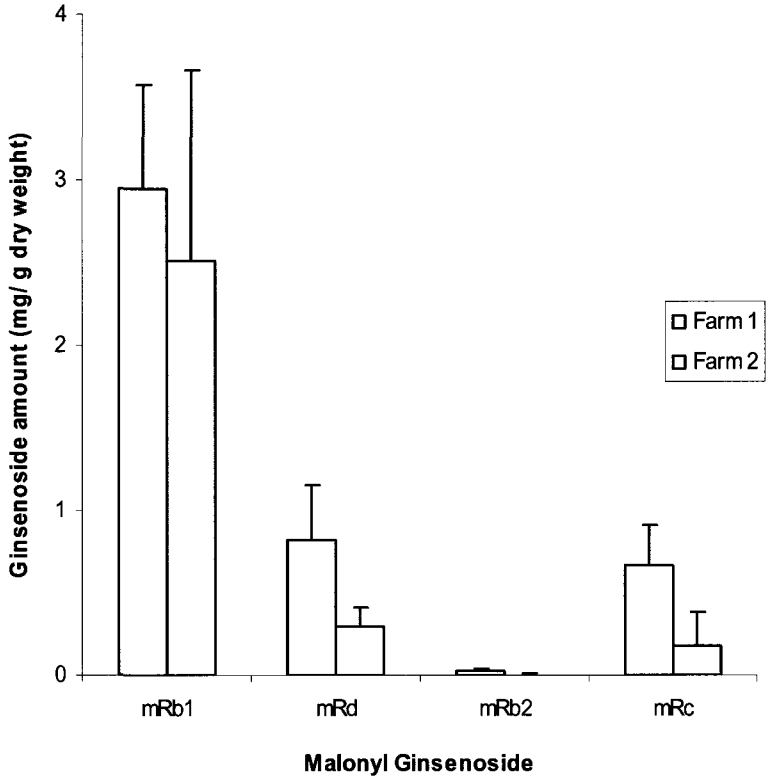

Figure 4. 4. Amount (mg/g dry weight) of malonyl ginsenosides in ginseng samples (n=5) grown at two Ontario ginseng farms. Amount of mRb2 differed significantly between farms as determined by ANOVA and Tukey's post-hoc analysis.

Table 4. 4. Percentage of total ginsenoside content made up by individual and total malonyl ginsenosides.

	Percentage of total ginsenoside content (%)				
Farm	mRb1	mRd	mRb2*	mRc	total malonyl ginsenosides
1	2.91- 9.9	0.20-4.15	0.01- 0.11	0-2.27	3.41- 16.45
2	1.6- 11.8	0.01-1.49	0-0.04	0-0.90	3.26-12.63

*Ginsenoside content differed significantly between farms (ANOVA and Tukey's post-hoc test, $p<0.5$)

Malonyl ginsenoside levels in these Ontario ginseng roots were similar to levels reported by Christensen et al. (2006), who observed levels of mRb1 between 2.8- 4.85 mg/g

fresh weight, levels of mRc between 0.17- 0.52 mg/g fresh weight, and levels of mRd between 0.79- 2.29 mg/g fresh weight when examining *P. quinquefolius* roots and root sections grown in Denmark. Malonyl ginsenoside content was a considerably lower however, in this study in comparison to values obtained by Court et al. (1996) who observed levels of mRb1 between 16.92-23.15 mg/g dry weight, levels of mRc between 2.09-2.13 mg/g dry weight, levels of mRd between 4.08-4.65 mg/g dry weight, and levels of mRb2 between 0.27-0.35 mg/g dry weight in *P. quinquefolius* roots obtained from an unspecified location. These studies support the present findings indicating a significant contribution of malonyl ginsenosides to total ginsenoside content.

Previous methods used to examine ginsenosides and malonyl ginsenosides are typically between 45- 118 minutes long. The significant reduction in analysis time to 25 minutes will allow for efficient analysis of large numbers of samples in routine analysis. Previous studies examining malonyl ginsenosides using MS have not quantified malonyl ginsenosides (Sloley et al., 2006;Kite et al., 2003) whereas other studies that have quantified malonyl ginsenosides by taking the difference in neutral ginsenosides before and after hydrolysis have not monitored ions associated with malonyl ginsenosides (Court et al., 1996;Christensen et al., 2006). This study combines traditional quantification methods with MS/MS which adds more confidence in the identification of malonyl ginsenosides and allows for confirmation of a reduction in malonyl ginsenosides after hydrolysis. Using MS/MS it is possible to tentatively identify compounds for which standards are unavailable and with further development, more ginsenosides may be identified.

4.3.3 HPLC-ELSD monosaccharide method validation

Calibration curves were produced from pure standard compounds from the concentrations within a linear range. An R^2= 0.99 was obtained for all compounds except galacturonic acid which produced an R^2 value of 0.90.

To assess the precision and accuracy of the method, variation in monosaccharide detection was assessed within and between days of analysis. Each day, each level was injected three times and variation is expressed as the percent coefficient of variation (%CV= standard deviation/mean x 100). Table 4. 5 shows intra- and interday variation. Variation was similar between days and in most cases was under 10%. Interday variation was generally under 10% with only 2 exceptions. Limit of detection was recorded at a peak height of 0.9 mV and limit of quantification at 2.7 mV for the Rezex RPM column analysis. For the Luna NH2 column analysis, limit of detection was recorded at a peak height of 0.45 mV and limit of quantification at 1.35 mV.

Table 4. 5. Inter- and Intraday variation for monosaccharides detected by HPLC-ELSD.

Compound	Concentration level	Intraday variation (%CV)			Interday variation
		Day 1	Day 2	Day 3	
Galacturonic Acid	Level 1	18.64	17.23	n.d.	7.87
	Level 2	12.95	6.47	10.60	12.95
	Level 3	6.19	11.60	3.36	1.59
	Level 4	23.18	10.09	4.81	8.33
	Level 5	4.03	13.44	11.19	3.51
	Level 6	12.55	7.31	6.75	9.81
	Level 7	9.69	1.46	14.94	1.06
	Level 8	7.53	3.67	3.59	1.01
Glucose	Level 1	1.26	0.39	0.36	2.50
	Level 2	0.26	0.65	0.16	5.13
	Level 3	0.51	0.41	0.63	5.45
	Level 4	0.04	0.94	0.72	4.60
	Level 5	0.91	0.49	1.03	4.48
	Level 6	0.15	0.97	n.d.	3.50
	Level 7	1.20	0.64	27.52	6.71
	Level 8	7.02	4.32	n.d.	0.32
Galactose	Level 1	0.88	0.27	0.06	1.84
	Level 2	1.10	0.24	0.30	5.12
	Level 3	0.73	0.25	0.29	5.36
	Level 4	0.33	0.80	0.74	4.56
	Level 5	0.45	0.75	0.55	4.68
	Level 6	1.06	0.47	n.d	2.94
	Level 7	2.23	1.50	18.58	5.66
	Level 8	2.29	3.33	n.d.	4.29
Arabinose	Level 1	2.16	1.81	0.84	5.11
	Level 2	0.96	0.92	1.50	6.35
	Level 3	0.69	0.91	0.72	7.09
	Level 4	1.79	2.26	0.05	6.14
	Level 5	1.13	1.97	0.20	6.56
	Level 6	2.18	2.19	n.d.	5.76
	Level 7	3.31	3.42	32.28	24.47

nd- not determined

4.3.4 HPLC-ELSD Ontario ginseng monosaccharide composition

Representative chromatograms showing individual monosaccharides in Ontario ginseng root samples are presented in Figure 4. 5. As shown in Table 4. 6, glucose was found to be the major neutral monosaccharide present in Ontario ginseng root with amounts ranging from 14.98- 23.59 mg/ g dry weight. Galactose and arabinose were present in similar amounts with galactose being present at levels of 0.48- 1.46 mg/g dry weight and arabinose being present at levels between 0- 0.79 mg/g dry weight. Galacturonic acid was also identified on Ontario ginseng samples at levels ranging from 5.54- 14.63 mg/g dry weight. A small peak at the position in which rhamnose elutes was detected in two of the five Ontario ginseng samples, however, it was present at levels below the limit of quantification. Mannose and xylose were also monitored but were not detected in the ginseng samples. Galacturonic acid has not previously been reported in the monosaccharide composition of *P. quinquefolius* but has been reported in *P. ginseng* and *P. notoginseng* polysaccharide extracts (Zhu et al., 2005;Zhang et al., 2009). In a previous HPLC study of *P. quinquefolius* monosaccharide components, glucose was found to be the major components, followed by galactose and arabinose and minor contributions of rhamnose, mannose, and fucose were also found but galacturonic acid was not assessed (Assinewe et al., 2002). Glucose has also been determined to be the most prominent monosaccharide in *P. ginseng* and *P. notoginseng*, followed by galacturonic acid and galactose (Zhu et al., 2005;Zhang et al., 2009).

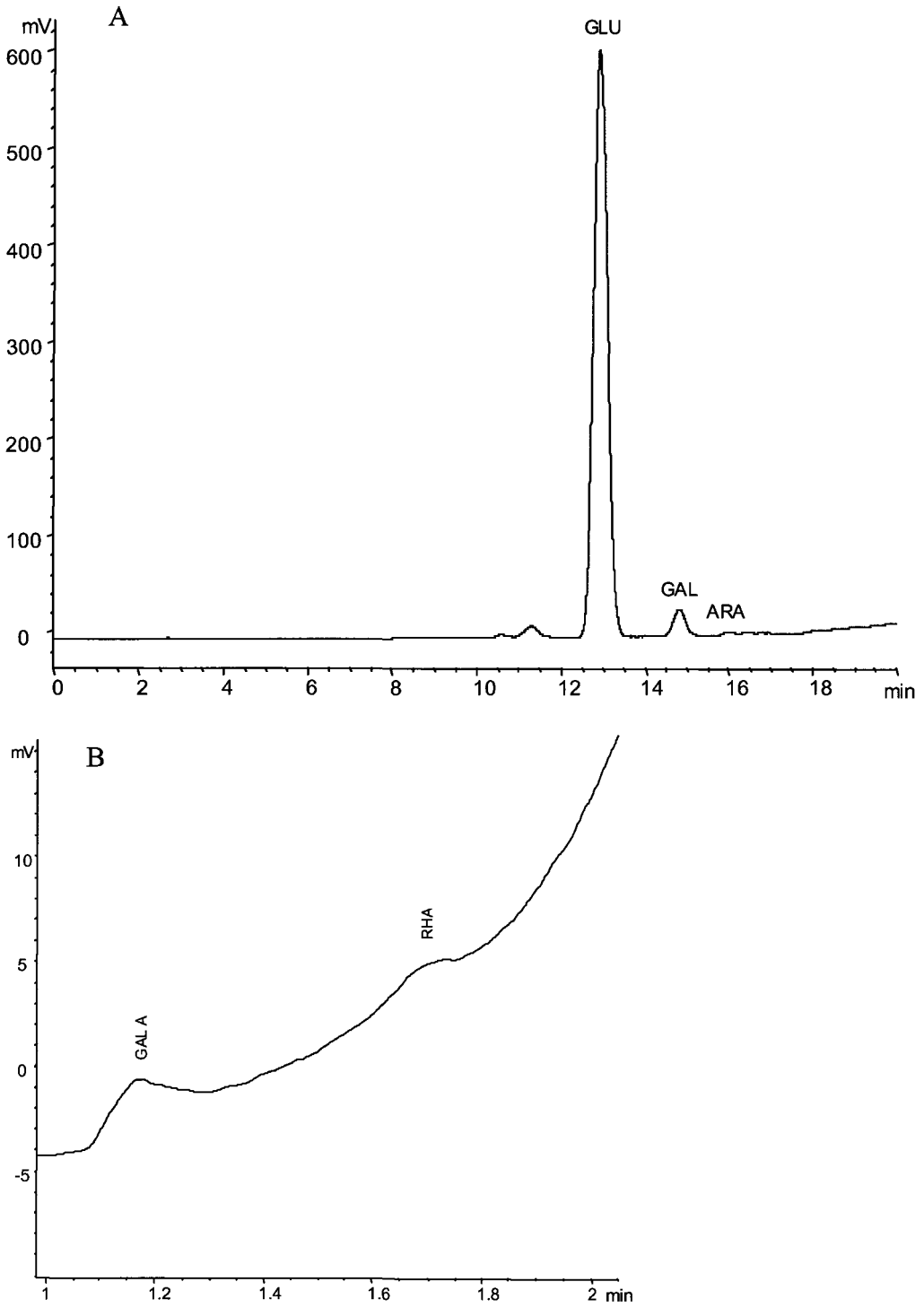

Figure 4. 5. Representative HPLC-ELSD Chromatograms of monosaccharide detected in Ontario ginseng, including glucose (Glu), galactose (Gal), arabinose (Ara) (A), galacturonic acid (Gal A) and rhamnose (Rha) (B).

Table 4. 6. Monosaccharide content (mg/g dry weight) in five Ontario ginseng samples. Each sample was tested in triplicate.

	Amount (mg/g dry weight)				
Sample	**GalA**	**Rhamnose**	**Glucose**	**Galactose**	**Arabinose**
1	9.64 (0.48)	blq	20.64 (1.14)	0.85 (0.02)	0
2	6.97 (.21)	0	22.59 (0.60)	0.56 (0.06)	0.75 (0.01)
3	9.15 (1.99)	0	14.98 (0.26)	0.46 (0.01)	0.26 (0.26)
4	14.63 (1.63)	0	15.07 (.50)	1.46 (0.31)	0.79 (0.01)
5	5.54 (0.40)	blq	23.59 (0.42)	0.48 (0.07)	0.75 (0.04)

blq- below the limit of quantification

The monosaccharide composition provides insight into the types of polysaccharides which may be found in *P. quinquefolius*. Polysaccharides structures in *P. quinquefolius* have not previously been thoroughly studied, though several polysaccharides have been isolated from *P. ginseng* and *P. notoginseng*. Glucose and galacturonic acid were the most prominent monosaccharides detected. Previously, polysaccharide fractions from *P. ginseng* with high levels of glucose have been determined to contain starch-like glucans and arabinogalactans and fractions with high levels of galacturonic acid have been shown to contain pectins with several linked galacturonic acid domains (Zhang et al., 2009). It is possible that polysaccharides similar to these may be present in Ontario ginseng though further work will have to be done to characterize the structures.

Commonly gas chromatography is used to characterize monosaccharide components. Although gas chromatography allows for good separation, quantification can be difficult as it is necessary to derivatize compounds to allow for analysis. The present method allows for the separation of 5 monosaccharides in ginseng, with the potential to identify 7 compounds (mannose and xylose were monitored but not detected in samples). This method is shorter than a previous method used to analyze monosaccharides in *P. quinquefolius* (Assinewe et

al., 2002), with a 30 minute total run time for both columns versus 60 minutes in the previous method. Furthermore, galacturonic acid has not previously been assessed in the monosaccharide composition of *P. quinquefolius*, but has been shown here to be present in considerable amounts. Although this method has improvements over previous methods, the broad peak shapes observed can result in quantification challenges and separation of multiple monosaccharides with a single column remains a challenge. This is one step toward further characterizing Ontario ginseng polysaccharides and introduces a new method that has the potential to be further developed and used for routine analysis.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

This study is the first to characterize malonyl ginsenosides in Ontario ginseng roots and introduces a new method for ginsenoside analysis. In comparison to previous methods for malonyl ginsenoside analysis, this method also allows for further confirmation of malonyl ginsenosides by identifying ions indicative of malonyl ginsenosides along with estimating the difference in neutral ginsenosides before and after sample hydrolysis. Malonyl ginsenoside were shown to account for up to 16.45% of total ginsenosides and should be included in analysis to obtain a more accurate estimation of total ginsenoside content.

Glucose was the major monosaccharide identified in Ontario ginseng root samples. Galacturonic acid, galactose, and arabinose were also detected in significant quantities. Few studies have examined monosaccharide components of *P. quinquefolius* and this is the first study to examine the composition in Ontario ginseng. To characterize polysaccharide structures in *P. quinquefolius* further work is being conducted to fractionate the crude

ginseng polysaccharide extract and isolate and identify polysaccharide structures using NMR.

4. 5 ACKNOWLEDGEMENTS

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CHAPTER 5

PREFACE

The work presented in this chapter was produced in collaboration with Kim Colson and Josh Hicks at Bruker BioSpin (Billerica Massachusetts). The study design was developed by myself, John Arnason, Kim Colson, and Josh Hicks and the NMR experiments were performed by Josh Hicks. To complement the characterization of Ontario ginseng's ginsenosides and monosaccharide components using chromatography methods, we wanted to generate metabolomic fingerprints of Ontario ginseng land races using NMR as a tool to examine the wide range of compounds present. The overall goal of this study was to develop a method to distinguish Ontario ginseng land races from one another, as these were not distinguished based on major ginsenoside markers in the study described in Chapter 2.

5.0 DISTINGUISHING GINSENG SPECIES AND ONTARIO GINSENG LAND RACES USING NMR AS A METABOLOMIC TOOL

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ABSTRACT

The use of NMR to distinguish and identify unique markers of five Ontario ginseng land races and two ginseng species was evaluated. Collecting 1D-NOESY, 1D-CPMG, JRES, COSY, and Ed-HSQC spectra, North American *P. quinquefolius* was distinguishable from Asian *P. ginseng* by PCA analysis. Of the five *P. quinquefolius* land races, two were distinguishable from one another and from the three remaining landraces, which clustered together in the PCA analysis. Land race 4 was distinguishable based on a higher level of anomeric glucose. Ginsenosides were identified in all samples and land race 5 was determined to be distinguishable based on a greater amount of methyl-ginsenoside sugars, indicating greater overall ginsenoside levels. The fact that some land races were distinguishable demonstrates the potential for this technique to be used to identify ginseng land races to the individual farm level, which will likely be improved over time as land races drift further apart.

5.1 INTRODUCTION

Ginseng cultivation in Ontario began over 100 years ago when the seed was cultivated from wild ginseng plants. Today Ontario ginseng (*Panax quinquefolius*) comprises several unimproved land races. A consistent seed stock has been maintained in these land races without mixing or introducing new seeds. In a previous HPLC-DAD study, ginsenoside content within and between Ontario ginseng land races was examined using 6 major ginsenosides (Rg1, Re, Rb1, Rc, Rb2, and Rd) as markers (McIntyre et al., 2010, in press). Using this method, it was clearly shown that significant variation in ginsenoside content does occur between land races, however, unique characteristics that would rapidly identify each land race based on these markers could not be identified. There is interest in identifying

unique characteristics of land races as the differences in phytochemical characteristics could lead to the development of unique cultivars with distinct activities. Furthermore, developing techniques to distinguish land races has standardization and quality control implications as it would display the possibility for cultivation location to be verified to the individual farm level.

Metabolomics, described as "the identification and quantification of all metabolites in a biological system" (Schripsema, 2010), is becoming an increasingly important characterization technique. Using ^{1}H NMR, wide-range profiling can be done as each signal corresponds to a hydrogen molecule in a compound, creating a profile with several thousand signals (Schripsema, 2010). This also makes structure identification possible and allows for a metabolomic fingerprint to be generated for each sample. Metabolomics research has the potential to play an important role in species identification and product standardization in the herbal and natural products market. By screening products and plants indiscriminately, wide range metabolite profiling can be achieved, resulting in robust and stringent quality control criteria (Lee et al., 2009).

There is interest in developing methods to rapidly distinguish between ginseng species. It is concerning when ginseng species are mislabelled as different ginseng species can have different therapeutic uses. For example, Asian and North American ginseng have been shown to have differing effects on acute glycemia (Sievenpiper et al., 2004) and on the vascular system (Sengupta et al., 2004). There are also commercial considerations, since there is a price differential between the species, with North American ginseng commanding a higher price. ^{1}H NMR could be applied to distinguish ginseng species as it provides rapid

data acquisition, vast chemical information, and generates a metabolomic fingerprint of each sample tested, without destruction of the sample. This technique has been successfully applied to differentiate between a variety of botanical species within the same genus and has also been used to differentiate between different cultivars and growing locations of the same plant species. ^{1}H NMR has been used to achieve distinction between three different *Echinacea* species (*E. angustifolia*, *E. pallida*, and *E. purpurea*) (Frédérich et al., 2010) and two different ginseng species (*P. ginseng* and *P. quinquefolius*) (Lee et al., 2009). This technique was successfully used to distinguish between several plants of the same species grown in China versus Korea (*Scutellaria baicalensis*, *Atractyloides japonica*, *Pueraria lobata*, and *Alisma orientale*) with greater than 90 % prediction accuracy (Kang et al., 2008a). ^{1}H NMR has also been used to distinguish between Chinese and Korean varieties of *Panax ginseng* (Kang et al., 2008b) and *Ganoderma lucidum* (Wen et al., 2010). Lee et al. (2009) were successful in applying ^{1}H NMR to separate and identify distinguishing features of *P. ginseng* cultivars (Yunpoong, Chunpoong, Keumpoong, and an unclassified local cultivar). There is great potential to apply this technique to quality control procedures to rapidly identify species, sample locations, and even specific varieties and cultivars.

This study focuses on the use of ^{1}H NMR to fingerprint and attempt to distinguish Ontario ginseng land races from one another as well as to distinguish North American and Asian ginseng from our collections to corroborate the Lee study (2009). This technique has not previously been used to distinguish land races of the same species and has implications in quality control, as source identification to a single farm could be made. Identifying distinguishing features of Ontario ginseng land races also has potential economic

implications as ginseng with different metabolomic profiles may ultimately be used for different medicinal purposes.

5.2 MATERIALS AND METHODS

5.2.1 NMR sample preparation

Twenty-one to twenty-five roots were collected from 1m x 1m plots at five farms in Norfolk County, Ontario with the following GPS coordinates:

43 08 13.05 N, 80 24 06.10 W

43 02 48.93 N, 80 23 45.15 W

42 55 43.95 N, 80 24 19.53 W

42 48 10.13 N, 80 31 55.20 W

42 51 12.64 N, 80 32 27.05 W

Samples were the same as those used in the study by McIntyre et al. (2010) and were extracted as described. Briefly, ground ginseng was extracted by sonication three times in 70% methanol, using 10 mL twice and 4mL on the third extraction. The phases were centrifuged and the supernatants were pooled and brought to 25 mL in a volumetric flask. Extracts were dried using a speed vac and lyophilized. 25mg of crude ginseng extract was dissolved in 1mL of deuterated dimethyl sulfoxide (DMSO-d6) by vortex for 25 seconds. Each sample was then centrifuged at 12000 x g for 30 seconds. 600 µL of supernatant was added to a NMR tube along with 1 µL of 300mM 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in DMSO-d6 solution.

5.2.2 NMR data acquisition

All NMR experiments were acquired on a Bruker AVANCE III spectrometer (600.13 MHz) at 298K with a Bruker 5 mm TCI CryoProbe. Spectra were collected and processed using the TopSpin 2.1 software (Bruker BioSpin, Billerica, USA). For all samples a suite of experiments were acquired as described here.

A standard 1-dimensional (1D) nuclear overhauser effect spectroscopy (NOESY) experiment (noesygp1d) was acquired using a mixing time of 10 ms with 64 scans, 64k data points and a sweep width of 20ppm. A relaxation delay of 1s was used to allow an acquisition time of ca. 6 minutes and 35 seconds. Data was processed with a line broadening factor of 0.3 Hz and zero filled to 64k data points.

A 1D- Carr - Purcell - Meiboom - Gill (CPMG) experiment was acquired using an echo time delay of 256 msec with 64 scans and 64k data points. A relaxation delay of 8s was used to allow an acquisition time of ca. 6 minutes and 35 seconds. The acquisition time for this spectrum was 7 minutes and 45 seconds.

A 2D J-Resolved (jresqf) spectrum was acquired to resolve couplings of overlapping peaks in the 1D data using the following acquisition parameters: 8k data points, with 40 increments, 2 scans, and an indirect sweep width of 78 Hz. The experiment time was 4 minutes and 7 seconds. The J-resolved spectrum was processed to an 8k by 128 data point spectrum using sine functions in both dimensions.

A ^{1}H correlation spectroscopy (COSY) experiment (cosygpppqf) was acquired with 8 scans, 4k data points, 16ppm sweep width, and 300 increments. A 2 s relaxation delay was used to give an experiment time of 1 hour 31 minutes. The COSY was processed to a 4K x 4K matrix.

A ^{1}H, ^{13}C- heteronuclear single quantum correlation (HSQC) experiment (hsqcedetgpsisp2.3) was acquired using 4k data points, 400 increments, 8 scans and a sweep width of 16ppm in the 1H dimension and 240pm in 13C dimension. A 1.5 s relaxation delay was used to give an experiment time of 1 hour 33 minutes.

NMR spectral referencing to the solvent peak at 2.51ppm and metabolite identification were conducted using the AMIX (Bruker BioSpin, Billerica, USA) software package with the an in-house natural products spectral base.

5.2.3 Statistical analysis

MATLAB (The MathWorks, Natick, Massachusetts, USA) was used for principal component analysis (PCA), confusion matrix and linear discriminant analysis (LDA).

5.2.4 NMR model generation

Models for each land race were generated using the 1D –CPMG spectra with LDA using 0.05 ppm buckets, pareto scaling, and with an explained variance from model center set to 0.9995. Five spectra were randomly removed before each model generation and projected back into the spectra looped for 20 Monte-Carlo simulations for model testing purposes.

5.3 RESULTS AND DISCUSSION

Ontario *P. quinquefolius* root extracts were clearly distinguishable from steamed *P. ginseng*, also known as Korean red ginseng, root extracts and extracts of *P. ginseng* commercial preparations by PCA analysis (Figure 5. 1). Commercial preparations labelled as *P. quinquefolius* were also assessed (shown in brown in Figure 5. 1) but were not separated in the PCA analysis, with different samples grouping with any of the three previously described groups. It is possible that some of these samples may have been mislabelled, or in the case where some *P. quinquefolius* commercial products are grouped with commercial preparations of *P. ginseng*, common excipients in the preparations may lead to more common metabolomic profiles.

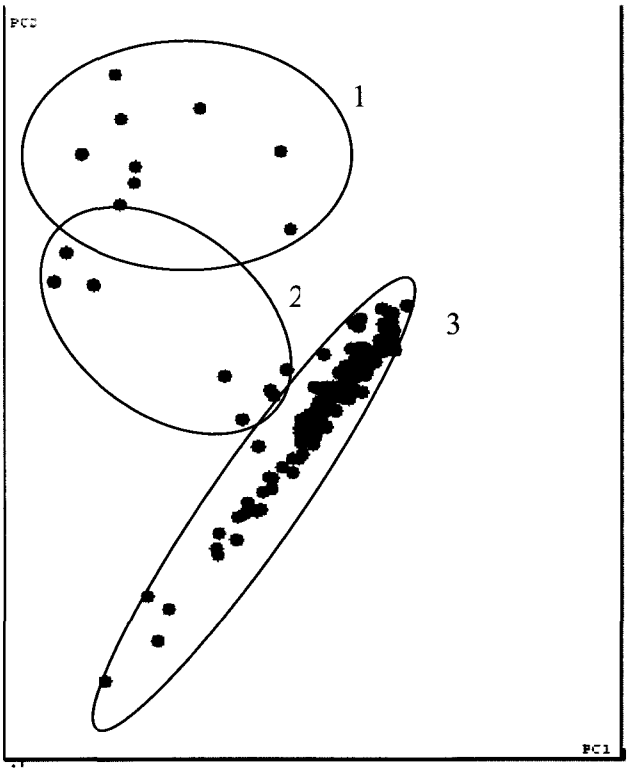

Figure 5. 1. PCA analysis separating steamed *Panax ginseng* root extracts (1- light blue), commercial preparations extracts of *Panax ginseng* (2- black) and *Panax quinquefolius* root extracts from five land races (3- purple, green, orange, red, blue).

Ginsenosides have generally been the most studied compounds in traditional metabolite profiling studies as ginsenoside content is often thought to be most telling of the medicinal properties. Ginsenosides Rb1 and Re were the only ginsenosides detected in amounts above the limit of quantification. Ginsenoside Rb1 was present in the range of 3.1-5.0 mM and Re was present in the range of 1.3-1.7 mM. Other ginsenosides were detected in amounts between 1-3 mM though standards would be required to confirm the particular ginsenosides.

Two land races were distinguished from one another (land races 4 and 5) and from the remaining three land races in the PCA analysis (Figure 5. 2). These land races showed higher than random predictability (20%) in the confusion matrix model with 47.8% predictability for land race 4 and 33.3% predictability for land race 5 (Figure 5. 3). An overlay representative of land races 4 and 5 is shown in Figure 5. 4a, displaying distinguishing factors. Land race 4 was distinguished by the presence of a greater amount of anomeric glucose (Figure 5. 4b). Land race 5 was distinguished from the others due to the fact that it had higher overall levels of methyl-ginsenoside sugars (Figure 5. 4c), indicating a greater level of total ginsenosides. The remaining three land races showed similar metabolic patterns.

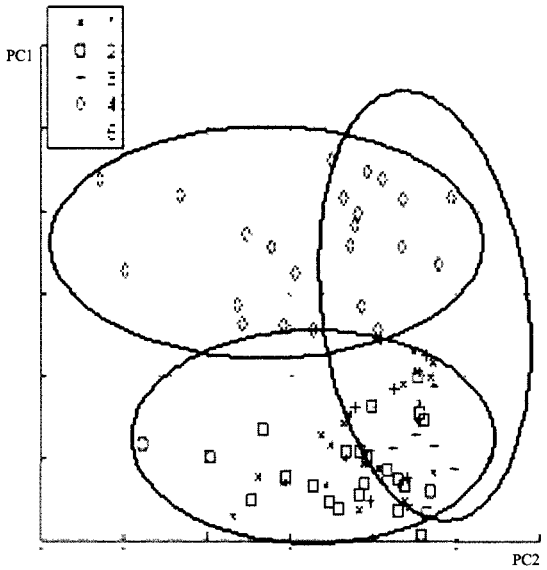

Figure 5. 2. PCA analysis of ginseng land races 1-5, showing the separation of landraces 4 and 5. The model was generated from LDA analysis using 0.05 ppm buckets, pareto scaled, with an explained variance from model center set to 0.9995. Five spectra were randomly removed before each model generation and projected back into the spectra looped for 20 Monte-Carlo simulations.

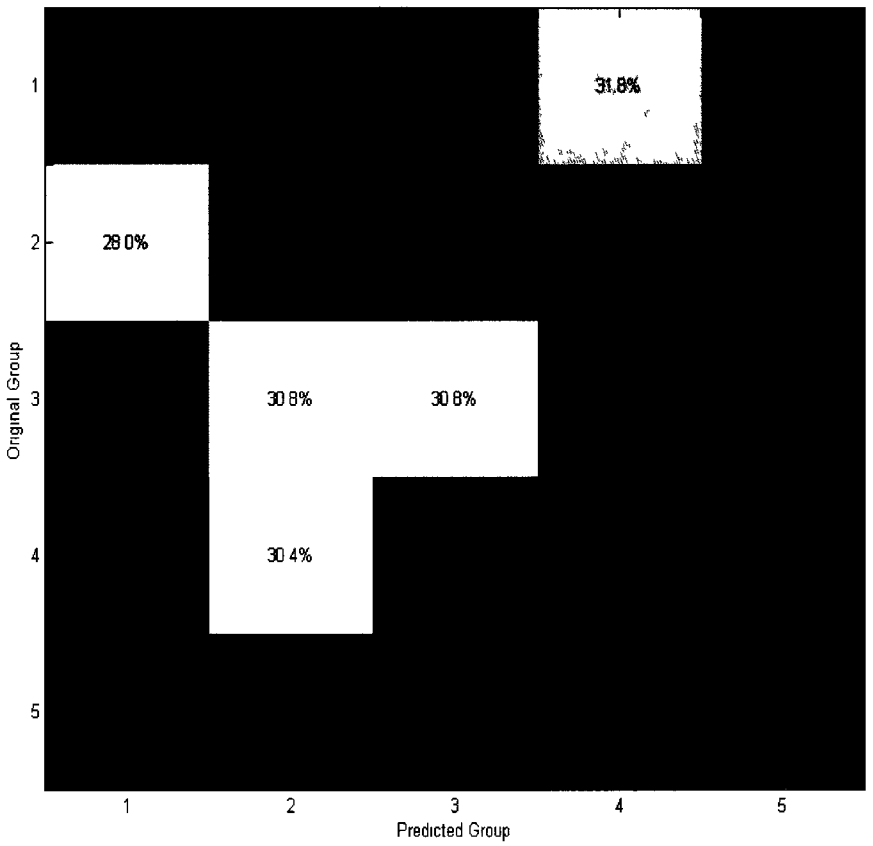

Figure 5. 3. Confusion matrix map generated to determine the distribution of categorization of samples based on models generated from the five different farms. Land races are represented as follows: 1 (red), 2 (green), 3 (blue), 4 (black), and 5 (yellow). The model was generated from LDA analysis using 0.05 ppm buckets, pareto scaled, with an explained variance from model center set to 0.9995. Five spectra were randomly removed before each model generation and projected back into the spectra looped for 20 Monte-Carlo simulations.

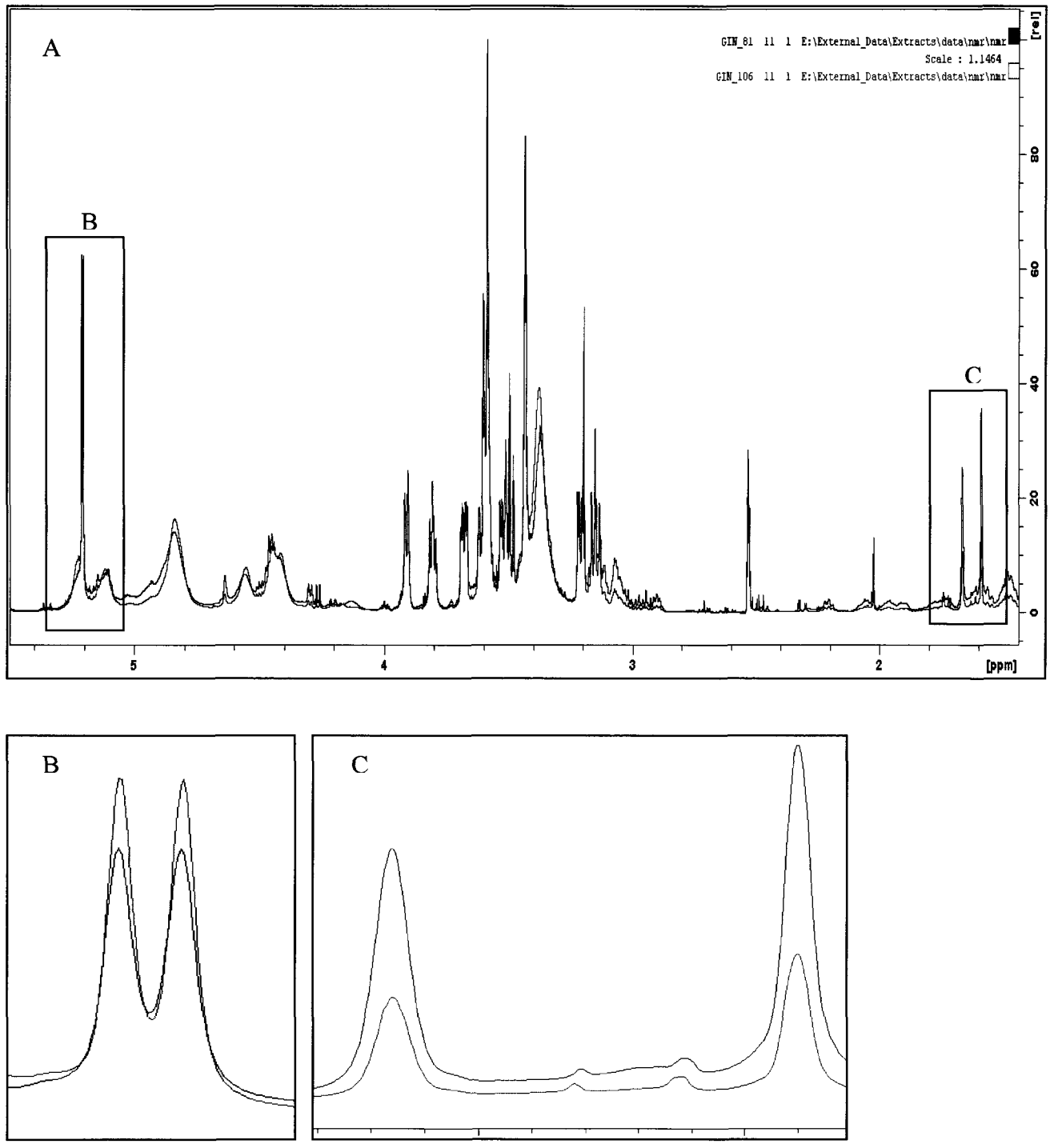

Figure 5. 4. Overlay of representative ginseng samples from land race 4 (blue) and land race 5 (red) (A) showing the signal at ca. 5.2 ppm (anomeric-glucose) is more intense in land race 4 (B) and the signals at ca. 1.58 and 1.67 ppm (methyl-ginsenoside sugars) are more intense in land race 5 (C).

In a recent study, ^{1}H NMR was successfully used to distinguish between cultivar varieties of *P. ginseng* (Lee et al., 2009). Cultivars are different from land races as cultivars are specifically selected for certain desirable characteristics and are consistent in reproducing those characters, whereas land races have adapted to particular environments, often with minimal human assistance, and have a broader range of phenotypes and genotypes. With the consistency and selectivity of cultivars, it is expected that they would be more distinct from one another than land races. In the case of *P. ginseng* cultivars, glucose and the amino acids asparagine, tyrosine, phenylalanine, and alanine were the most significant distinguishing factors (Lee et al., 2009). Amino acids were also assessed in this study but not found to distinguish land races. Although it was not possible to differentiate all five land races, two land races did show distinct metabolic patterns. This shows the potential for land races grown at different farms in relatively close proximity (less than 50 kilometres) to begin to drift apart over a period of approximately 50 years. The fact that some distinguishing features were found shows that plants can adapt to their local environments which can be revealed as changes in metabolic profile. It is likely that over the next several decades, the land races will drift further apart and be more easily distinguishable. Future studies could examine the land race metabolome over several years to examine this possibility.

5.4 CONCLUSIONS AND FUTURE DIRECTIONS

This study shows the potential for NMR to be used to distinguish between ginseng species and the potential to distinguish different land races of the same species. This technique could be used to quickly identify mislabelled products and could be developed further to rapidly confirm growing locations and identify ginseng that may come from farms with identified contamination, or on the contrary, identify ginseng from farms with highly

desirable characteristics. Ultimately NMR allows for very comprehensive screening of natural products and its use could lead to stricter quality control criteria.

5.5 ACKNOWLEDGEMENTS

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CHAPTER 6

6.0 GENERAL DISCUSSION

6.1 CLAIMS TO ORIGINALITY

This thesis provides a thorough characterization of Ontario ginseng assessing both phytochemistry and biological activities and is the first comparative assessment of ginseng land races. Ginsenoside variation has never before been examined in Ontario ginseng land races and it was clearly shown that significant variation does occur both within and between land races. Furthermore, variation in micropropagated clonal breeding lines supports the potential for micropropagation to be used to create a variety of Ontario ginseng lines with unique ginsenoside profiles. The production of lines with reproducible phytochemical and medicinal properties would create a new market in the North American ginseng industry.

A correlation was observed between CYP3A4 inhibition and ginsenoside content. Although biological activities of ginseng have been broadly studied, activity is rarely assessed in relation to ginsenoside content. This correlation demonstrates the importance of considering phytochemistry in relation to ginsenoside content, especially since there is high variation in ginsenoside content between roots.

Malonyl ginsenosides were shown to account for a significant percentage of total ginsenoside content. This is the first study to examine malonyl ginsenosides in Ontario ginseng and shows that malonyl ginsenosides should be included in analysis of total ginsenoside content. The LC/MS/MS method developed to examine this broader range of

ginsenosides provides a shorter analysis time and the collection of fragmentation ion data, allowing compound identification and quantification.

This is the first report of monosaccharide composition in Ontario ginseng as determined by HPLC-ELSD analysis. The monosaccharide composition will provide some insight into the types of polysaccharides that may be present in Ontario ginseng.

Although cultivars of the same species (*P. ginseng*) have been distinguished using ^{1}H NMR (Lee et al., 2009), this is the first study showing the potential for separation of ginseng land races (*P. quinquefolius*) using this metabolomic technique. This has significant quality control implications as sample location could be verified to the farm level as unique markers are identified.

6.2 COMPARISON TO PUBLISHED LITERATURE

In comparison to other *P. quinquefolius* populations (Assinewe et al., 2003;Schlag and McIntosh, 2006;Lim et al., 2005;Li et al., 1996), Ontario ginseng was found to have greater total ginsenosides (Table 6. 1). This could be attributed to growing location, however, differences in production and extraction methods, root diameter, and root age must also be considered as well as the limited dataset. Furthermore, Ontario ginseng land races generally had a greater proportion of ginsenoside Rd in comparison to other *P. quinquefolius* populations. Variation in ginsenoside content and composition shown in this study and published literature shows the importance of considering multiple roots in estimating ginsenoside content. Variation was also observed in ginseng breeding lines, and the use of

micropropagation was demonstrated to be an effective method to produce ginseng roots with similar levels to those in cultivated and wild roots of the same age (Table 6. 1).

Table 6. 1. Comparison of mean total ginsenoside content in *P. quinquefolius* roots from different populations and produced by varying methods.

Study	Mean total ginsenosides (%w/w)	Production method	Location	Age
This study (land races)	7.7	cultivated	Ontario	4
This study (4 yr micropropagated)	7.5	micropropagated	Ontario	4
Li et al. (1996)	3	cultivated	British Columbia	4
Assinewe et al. (2003)	5.4	cultivated and wild	Ontario, Quebec, Maine, Vermont, Wisconsin	4
Schlag et al. (2006)	2.4	cultivated and wild	Maryland	4
This study (1 yr micropropagated)	3.7	micropropagated	Ontario	1
Qu et al. (2008)	2.7	cultivated	Jilin Province, China	1

P. quinquefolius has been reported to possess a variety of anti-diabetic effects, and its blood glucose lowering effects have been well studied (Dascalu et al., 2007;Vuksan et al., 2000a;Vuksan et al., 2001;Vuksan et al., 2000b). Increased levels of advanced glycation endproducts and oxidation activity are associated with diabetic complications though Ontario *P. quinquefolius* did not exhibit strong anti-glycation or antioxidant activity in this study. In an *in vivo* study, advanced glycation endproducts were significantly reduced in the renal tissue of diabetic rats with the treatment of steamed *P. quinquefolius* though untreated *P. quinquefolius* showed no effect (Hyun et al., 2007). The steaming process initiates reactions forming new ginsenosides unique to steamed ginseng and the value of these ginsenosides in reducing diabetic complications could be further examined. *P. ginseng* has demonstrated antioxidant activity *in vivo*, reducing the expression of protein associated with oxidative

stress in the renal tissue of diabetic rats and increasing the levels of endogenous antioxidant glutathione in astrocyte cells under oxidative stress (Ki et al., 2006;Naval et al., 2007). *P. quinquefolius* was shown to increase the levels of Nrf2, a transcription factor of endogenous antioxidant defences, in a cardiac cell model (Li et al., 2010). However, in a diabetic rat model, *P. quinquefolius* failed to reduce oxidative stress in the renal tissues (Hyun et al., 2007). Few *in vivo* or clinical studies have examined the effects of *P. quinquefolius* on diabetic complications, including anti-glycation or antioxidant activity. Although low activity was observed in this *in vitro* study, there is some evidence that ginseng may exert its effects by influencing endogenous defence systems, which should be further monitored *in vivo*.

A potential herb-drug interaction was shown as ginseng significantly inhibited CYP3A4 and this activity was related to ginsenoside content. Although few studies have examined ginsenoside extracts with natural ginsenoside composition, previous studies have also shown an inhibition of CYP3A4 as well as CYP2C9, which was not observed in the present study, by individual ginsenosides *in vitro* (Hao et al., 2008;He and Edeki, 2004). Furthermore, Lui et al. (2006) reported that ginsenoside metabolites may play a more important role in cytochrome P450 enzyme inhibition than natural un-metabolized ginsenosides. Ethridge et al. observed inhibition of CYP3A4 by 2 individual ginsenosides but not *P. ginseng* extract. These results combined suggest that there is a need to study ginseng extracts with naturally occurring ginsenoside profiles, in products actually taken by consumers, in a clinical setting, examining both the effects of ginsenosides and ginsenoside metabolites.

Malonyl ginsenoside content was considerably lower here than what was found in a previous study by Court et al. (Court et al., 1996) but similar in amount to *P. quinquefolius* roots cultivated in Denmark (Table 6. 2). The results found here support the statements by Awang et al. (2000) that failure to include malonyl ginsenosides in analysis can lead to an underestimation of total ginsenoside content. Two other studies have used MS to examined malonyl ginsenosides in *P. quinquefolius* using a similar approach to choose ions to monitor malonyl ginsenosides as the present study (Sloley et al., 2006;Kite et al., 2003). However, these studies did not quantify malonyl ginsenosides. The present study combines MS detection of malonyl ginsenosides before and after hydrolysis along with traditional methods to quantify malonyl ginsenosides by taking the difference in neutral ginsenosides before and after sample hydrolysis.

Table 6. 2. Range of malonyl ginsenoside content in different populations of P. *quinquefolius*.

	Range of Ginsenoside content (mg/g)				
Population	**mRb1**	**mRd**	**mRb2**	**mRc**	**Reference**
Farm 1	0.97- 4.36	0.10- 1.82	0.01- 0.05	0- 1.13	Present study
Farm 2	0.56- 6.84	0.01- 0.51	0- 0.02	0- 0.67	Present study
Denmark	2.86- 4.85	0.79- 2.29	n/a	0.17-0.52	Christensen et al. (2009)
Unspecified	16.92-23.15	4.08-4.65	0.27-0.35	2.09- 2.13	Court et al. (1996)

Similar to previous reports of monosaccharide composition in *P. quinquefolius* and *P. ginseng*, glucose was the major monosaccharide present. Galactose, arabinose, and galacturonic acid were also detected and this is the first report of galacturonic acid in Ontario ginseng. Monosaccharide composition in comparison to another *P. quinquefolius*

polysaccharide extract as well as a *P. notoginseng* polysaccharide extract and *P. ginseng* polysaccharide fractions is presented in Table 6. 3.

Table 6. 3. Monosaccharide composition reported in ginseng roots.

	Monosaccharide (%)							
Study	Glucose	Galacturonic Acid	Galactose	Arabinose	Rhamnose	Mannose	Species	Detection method
This study	47.2- 77.7	18.24- 45.79	1.81- 4.57	0- 2.47	blq	nd	*P. quinquefolius*	HPLC-ELSD
Assinewe et al. (2003)	85.09	na	7.48	5.89	0.79	0.41	*P. quinquefolius*	HPLC-UV
Zhu et al. (2005)	75	11	11	3	blq	blq	*P. notoginseng*	GC-MS
Zhang et al. (2009)	1.3- 95.3	1.8- 92.1	3.3- 56.2	1.3- 40.9	0.2- 4.1	0.2- 3.6	*P. ginseng*	HPLC-UV

blq- below limit of quantification
nd- not detected
na- not assessed

It is clear that there is variation in ginsenoside profiles between locations and also that ginsenoside content can be correlated with biological activity. This variability can make standardization difficult so there is value in quickly being able to distinguish populations for quality control purposes. Due to high variation in ginsenoside content within Ontario ginseng land races, there were no clear identifying ginsenoside markers that would distinguish land races from one another based on HPLC profiles. Furthermore, along with malonyl ginsenosides and polysaccharides, there is a wide range of compounds in Ontario ginseng and single chromatographic methods cannot examine broad ranges of different types of compounds. Taking a metabolomics approach, ^{1}H NMR successfully distinguished Ontario ginseng land races from steamed *P. ginseng* and *P. ginseng* commercial products. *P. ginseng* was also distinguished from *P. quinquefolius* in a study by Lee et al. (2009) assessing samples cultivated in China. ^{1}H NMR is a robust technique that can distinguish ginseng species even under different conditions and examining samples from varying locations. *P.*

ginseng cultivars have been distinguished using ^{1}H NMR (Lee et al., 2009), but *P. quinquefolius* cultivars or land races had not been previously assessed and this study showed that some land races were distinct. It is likely that land races will continue to drift apart over time.

Overall, Ontario ginseng shows similarities to other populations of *P. quinquefolius* in the types of ginsenosides and monosaccharides present, however Ontario ginseng does show some originality in composition of these components compared to previously published reports. The wide variety in ginsenoside content in Ontario ginseng shows the potential to develop unique cultivars with stable and predictable ginsenoside composition and activity, and ^{1}H NMR could be a useful tool in the standardization of a variety of ginseng products with unique profiles. This characterization of Ontario ginseng will allow for the further development and marketing of Ontario ginseng products.

6.3 FUTURE RESEARCH

It is possible that unique activities could be discovered for ginseng land races or breeding lines with distinct ginsenoside composition, leading to a variety of ginseng cultivars with specific uses. Biological activity should be further investigated in *in vivo* and clinical studies, especially in the case of ginseng and CYP3A4 where inhibition was clearly shown to be related to ginsenoside content.

Techniques such as fractionation to generate polysaccharides of different molecular weights, combined with LC/MS/MS and NMR could be used to attempt to identify polysaccharide structures as well as identify potential unknown compounds in *P.*

quinquefolius. This would add significantly to the characterization of Ontario *P. quinquefolius*.

Ontario ginseng land races are beginning to drift a part and have unique characteristics as shown in the ^{1}H NMR study. This is a unique opportunity to monitor the changes in a new crop developed from a wild species. A long term study could be conducted to examine Ontario ginseng land races over several years to observe if the land races will change over time.

This thesis presents a new approach to characterizing of Ontario ginseng. The results from this study and future studies will contribute to the validation of Ontario ginseng and contribute to the value of this important medicinal crop.

REFERENCES

Ahmed, N., 2005. Advanced glycation endproducts - Role in pathology of diabetic complications. Diabetes Research and Clinical Practice 67, 3-21.

Aithal, G.P., Day, C.P., Kesteven, P.J.L., Daly, A.K., 1999. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. Lancet 353, 717-719.

Allen, J.D., McLung, J., Nelson, A.G., Welsch, M., 1998. Ginseng supplementation does not enhance healthy young adults' peak aerobic exercise performance. Journal of the American College of Nutrition 17, 462-466.

Anderson, R.C., Fralish, J.S., Armstrong, J.E., 1993. The ecology and biology of *Panax quinquefolium* L. (Araliaceae) in Illinois. American Midland Naturalist 129, 357-372.

Arnason, J.T., Habda, R.J., Johns, T., 1981. Use of plants for food and medicine by Native Peoples of eastern Canada. Canadian Journal of Botany 59, 2189-2325.

Assinewe, V.A., Baum, B.R., Gagnon, D., Arnason, J.T., 2003. Phytochemistry of wild populations *of Panax quinquefolius* L. (North American ginseng). Journal of Agricultural and Food Chemistry 51, 4549-4553.

Assinewe, V.A., Arnason, J.T., Aubry, A., Mullin, J., Lemaire, I., 2002. Extractable polysaccharides of *Panax quinquefolius* L. (North American ginseng) root stimulate TNFα production by alveolar macrophages. Phytomedicine 9, 398-404.

Awang, D.V.C., 2000. The neglected ginsenosides of North American ginseng (*Panax quinquefolius* L.). Journal of Herbs, Spices and Medicinal Plants 7, 103-109.

Bae, J.W., Lee, M.H., 2004. Effect and putative mechanism of action of ginseng on the formation of glycated hemoglobin in vitro. Journal of Ethnopharmacology 91, 137-140.

Bahrke, M.S. and Morgan, W.P., 1994. Evaluation of the ergogenic properties of ginseng. Sports Medicine 18, 229-248.

Bahrke, M.S., Morgan, W.P., 2000. Evaluation of the ergogenic properties of ginseng: an update. Sports Medicine 29, 113-133.

Bai, D., Brandle, J., Reeleder, R., 1997. Genetic diversity in North American ginseng (*Panax quinquefolius* L.) grown in Ontario detected by RAPD analysis. Genome 40, 111-115.

Biondo, P.D., Goruk, S., Ruth, M.R., O'Connell, E., Field, C.J., 2008. Effect of CVT-E002™ (COLD-fX®) versus a ginsenoside extract on systemic and gut-associated immune function. Int. Immunopharmacology 8, 1134-1142.

Blumenthal, M. (ed), 2003. The ABC Clinical Guide to Herbs. American Botanical Council. Austin, TX.

Burkhart, E.P., Jacobson, M.G., 2009. Transitioning from wild collection to forest cultivation of indigenous medicinal forest plants in eastern North America is constrained by lack of profitability. Agrofor. Syst. 76, 437-453.

Carlson, A.W., 1986. Ginseng: America's Botanical Drug Connection to the Orient. Economic Botany 40, 233-233-249.

Chan, S.W., Li, S., Kwok, C.Y., Benzie, I.F.F., Szeto, Y.T., Guo, D.J., He, X.P., Yu, P.H.F., 2008. Antioxidant activity of Chinese medicinal herbs. Pharmaceutical Biology 46, 587-595.

Chan, T.W.D., But, P.P.H., Cheng, S.W., Kwok, I.M.Y., Lau, F.W., Xu, H.X., 2000. Differentiation and authentication of *Panax ginseng, Panax quinquefolius*, and ginseng products by using HPLC/MS. Analytical Chemistry 72, 1281-1287.

Chang, T.K.H., 2004. Drug-metabolizing enzymes and p-glycoprotein, in Handbook of Drug-Nutrient Interactions (Boullata JI and Armenti VT eds) pp 43-67, Humana Press, New Jersey.

Charron, D., Gagnon, D., 1991. The demography of northern populations of *Panax quinquefolium* (American ginseng). Journal of Ecology 79, 431-445.

Chen, J., Xie, M., Fu, Z., Lee, F.S.-., Wang, X., 2007. Development of a quality evaluation system for *Panax quinquefolium*. L based on HPLC chromatographic fingerprinting of seven major ginsenosides. Microchemical Journal 85, 201-208.

Chinese Pharmacopoeia Commission, 2005. Pharmacopoeia of the People's Republic of China. Chinese Pharmacopoeia Commission, Beijing.

Cho, W.C.S., Chung, W.-., Lee, S.K.W., Leung, A.W.N., Cheng, C.H.K., Yue, K.K.M., 2006. Ginsenoside Re of Panax ginseng possesses significant antioxidant and antihyperlipidemic efficacies in streptozotocin-induced diabetic rats. European Journal of Pharmacology 550, 173-179.

Choi, K., 2008. Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C A Meyer. Acta Pharmacologica Sinica 29, 1109-1118.

Christensen, L.P., 2008. Chapter 1 Ginsenosides. Chemistry, Biosynthesis, Analysis, and Potential Health Effects. Advances in Food and Nutrition Research 55, 1-99.

Christensen, L.P., Jensen, M., Kidmose, U., 2006. Simultaneous determination of ginsenosides and polyacetylenes in American ginseng root (*Panax quinquefolium* L.) by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 54, 8995-9003.

Court, W.A., Hendel, J.G., Elmi, J., 1996. Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. Journal of Chromatography A 755, 11-17.

Dascalu, A., Sievenpiper, J.L., Jenkins, A.L., Stavro, M.P., Leiter, L.A., Arnason, J.T., Vuksan, V., 2007. Five batches representative of Ontario-grown American ginseng root produce comparable reductions of postprandial glycemia in healthy individuals. Canadian Journal of Physiology and Pharmacology 85, 856-864.

de Andrade, E., de Masquita, A.A., de Almeida Claro, J., de Andrade, P.M., Ortiz, V., Paranhos, M., Srougi, M., 2007. Study of the efficacy of Korean Red Ginseng in the treatment of erectile dysfunction. Asian Journal of Andrology 9, 241-244.

Dong, T.T.X., Cui, X.M., Song, Z.H., Zhao, K.J., Ji, Z.N., Lo, C.K., Tsim, K.W.K., 2003. Chemical assessment of roots of *Panax notoginseng* in China: Regional and seasonal variations in its active constituents. Journal of Agricultural and Food Chemistry 51, 4617-4623.

Ellis, J.M., Reddy, P., 2002. Effects of Panax ginseng on quality of life. The Annals of Pharmacotherapy 36, 375-379.

Engels, H.J., Kolokouri, I., Cieslak, T.J., Wirth, J.C., 2001. Effects of ginseng supplementation on supramaximal exercise performance and short-term recovery. Journal of Strength and Conditioning Research 15, 290-295.

Etheridge, A.S., Black, S.R., Patel, P.R., So, J., Mathews, J.M., 2007. An in vitro evaluation of cytochrome P450 inhibition and p-glycoprotein interaction with goldenseal, ginkgo biloba, grape seed, milk thistle, and ginseng extracts and their constituents. Planta Medica 73, 731-741.

Farsi, D.A., Harris, C.S., Reid, L., Bennett, S.A.L., Haddad, P.S., Martineau, L.C., Arnason, J.T., 2008. Inhibition of non-enzymatic glycation by silk extracts from a Mexican land race and modern inbred lines of maize (*Zea mays*). Phytotherapy Research 22, 108-112.

Foster, S., 1996. American ginseng *Panax quinquefolius*. American Botanical Council Botanical Series 308.

Frédérich, M., Jansen, C., De Tullio, P., Tits, M., Demoulin, V., Angenot, L., 2010. Metabolomic analysis of *Echinacea* spp. by1H nuclear magnetic resonance spectrometry and multivariate data analysis technique. Phytochemical Analysis 21, 61-65.

Fugh-Berman, 2000. Herb-drug interactions. Lancet 355, 134-138.

Fujimoto, Y., Hongcheng, W., Kirisawa, M., Satoh, M., Takeuchi, N., 1992. Acetylenes from *Panax quinquefolium*. Phytochemistry 31, 3499-3501.

Fujimoto, Y., Satoh, M., Takeuchi, N., Kirisawa, M., 1991. Cytotoxic acetylenes from *Panax quinquefolium*. Chemical and Pharmaceutical Bulletin 39, 521-523.

Hao, M., Zhao, Y., Chen, P., Huang, H., Liu, H., Jiang, H., Zhang, R., Wang, H., 2008. Structure-activity relationship and substrate-dependent phenomena in effects of ginsenosides on activities of drug-metabolizing P450 enzymes. PLoS ONE 3, e2697.

Harbilas, D., Martineau, L.C., Harris, C.S., Adeyiwola-Spoor, D.C.A., Saleem, A., Lambert, J., Caves, D., Johns, T., Prentki, M., Cuerrier, A., Arnason, J.T., Bennett, S.A.L., Haddad, P.S., 2009. Evaluation of the antidiabetic potential of selected medicinal plant extracts from the Canadian boreal forest used to treat symptoms of diabetes: Part II. Canadian Journal of Physiology and Pharmacology 87, 479-492.

Harris, C.S., Lambert, J., Saleem, A., Coonishish, J., Martineau, L.C., Cuerrier, A., Haddad, P.S., Arnason, J.T., Bennett, S.A.L., 2008. Antidiabetic activity of extracts from needle, bark, and cone of *Picea glauca*: Organ-specific protection from glucose toxicity and glucose deprivation. Pharmaceutical Biology 46, 126-134.

He, C.N., Gao, W.W., Yang, J.X., Bi, W., Zhang, X.S., Zhao, Y.J., 2009. Identification of autotoxic compounds from fibrous roots of *Panax quinquefolium* L. Plant Soil 318, 63-72.

He, N., Edeki, T., 2004. The inhibitory effects of herbal components on CYP2C9 and CYP3A4 catalytic activities in human liver microsomes. American Journal of Therapeutics 11, 206-212.

Henderson, G.L., Harkey, M.R., Gershwin, M.E., Hackman, R.M., Stern, J.S., Stressor, D.M., 1999. Effects of ginseng components on cDNA-expressed cytochrome P450 enzyme catalytic activity. Life Sciences 65, 209-214.

Hu, C., Kitts, D.D., 2001. Free radical scavenging capacity as related to antioxidant activity and ginsenoside composition of Asian and North American Ginseng extracts. JAOCS, Journal of the American Oil Chemists' Society 78, 249-255.

Hwang, S.-., Son, D.J., Kim, I.-., Kim, D.-., Sohn, S.-., Lee, J.-., Kim, S.-., 2008. Korean red ginseng attenuates hypercholesterolemia-enhanced platelet aggregation through suppression of diacylglycerol liberation in high-cholesterol-diet-fed rabbits. Phytotherapy Research 22, 778-783.

Hyun, Y.K., Ki, S.K., Yamabe, N., Nagai, R., Yokozawa, T., 2007. Protective effect of heat-processed American ginseng against diabetic renal damage in rats. Journal of Agricultural and Food Chemistry 55, 8491-8497.

Janetzki, K. and Morreale, A.P., 1997. Probable interaction between warfarin and ginseng. American Journal of Health-System Pharmacy 54, 692-693.

Ji, Q.C., Harkey, M.R., Henderson, G.L., Eric Gershwin, M., Stern, J.S., Hackman, R.M., 2001. Quantitative determination of ginsenosides by high-performance liquid chromatography-tandem mass spectrometry. Phytochemical Analysis 12, 320-326.

Jones, B.D. and Runikis, A.M., 1987. Interaction of ginseng with phenelzine. Journal of Clinical Psychopharmacology 7, 201-202.

Kang, J., Choi, M., Kang, S., Hyuk, N.K., Wen, H., Chang, H.L., Park, M., Wiklund, S., Hyo, J.K., Sung, W.K., Park, S., 2008a. Application of a 1H nuclear magnetic resonance (NMR) metabolomics approach combined with orthogonal projections to latent structure-discriminant analysis as an efficient tool for discriminating between Korean and Chinese herbal medicines. Journal of Agricultural and Food Chemistry 56, 11589-11595.

Kang, J., Lee, S., Kang, S., Kwon, H.N., Park, J.H., Kwon, S.W., Park, S., 2008b. NMR-based metabolomics approach for the differentiation of ginseng (*Panax ginseng*) roots from different origins. Archives of Pharmacal Research 31, 330-336.

Keum, Y., Park, K., Lee, J., Chun, K., Park, J.H., Lee, S.K., Kwon, H., Surh, Y., 2000. Antioxidant and anti-tumor promoting activities of the methanol extract of heat-processed ginseng. Cancer Letters 150, 41-48.

Ki, S.K., Hyun, Y.K., Yamabe, N., Nagai, R., Yokozawa, T., 2006. Protective effect of sun ginseng against diabetic renal damage. Biological and Pharmaceutical Bulletin 29, 1678-1684.

Kite, G.C., Howes, M.-.R., Leon, C.J., Simmonds, M.S.J., 2003. Liquid chromatography/mass spectrometry of malonyl-ginsenosides in the authentication of ginseng. Rapid Communications in Mass Spectrometry 17, 238-244.

Kitts, D.D., Popovich, D.G., Hu, C., 2007. Characterizing the mechanism for ginsenoside-induced cytotoxicity in cultured leukemia (THP-1) cells. Canadian Journal of Physiology Pharmacology 85, 1173-1183.

Kitts, D.D., Wijewickreme, A.N., Hu, C., 2000. Antioxidant properties of a North American ginseng extract. Molecular and Cellular Biochemistry 203, 1-10.

Kirchheiner, J. and Brockmoller, J., 2005. Clinical consequences of cytochrome P450 2C9 polymorphisms. Clinical Pharmacology and Therapeutics 77, 1-16.

Lee, E., Rustem, S., Weljie, A.M., Vogel, H.J., Facchini, P.J., Park, S., Kim, Y., Yang, T., 2009. Quality assessment of ginseng by 1H NMR metabolite fingerprinting and profiling analysis. Journal of Agricultural and Food Chemistry 57, 7513-7522.

Lee, M., Hwang, J., Kim, S., Yoon, S., Kim, M., Yang, H.J., Kwon, D.Y., 2010. Ginsenoside Rc, an active component of Panax ginseng, stimulates glucose uptake in C2C12 myotubes through an AMPK-dependent mechanism. Journal of Ethnopharmacology 127, 771-776.

Lee, S.H., Ahn, Y.M., Ahn, S.Y., Doo, H.K., Lee, B.C., 2008. Interaction between warfarin and Panax ginseng in ischemic stroke patients. Journal of Alternative and Complementary Medicine 14, 715-721.

Leung, K.W., Yung, K.K.L., Mak, N.K., Chan, Y.S., Fan, T.P., Wong, R.N.S., 2007. Neuroprotective effects of ginsenoside-Rg1 in primary nigral neurons against rotenone toxicity. Neuropharmacology 52, 827-835.

Lewis, W.H., Zenger, V.E., 1983. Breeding systems and fecundity in the American ginseng, *Panax quinquefolium* (Araliaceae). American Journal of Botany 70, 466-468.

Li, J., Ichikawa, T., Jin, Y., Hofseth, L.J., Nagarkatti, P., Nagarkatti, M., Windust, A., Cui, T., 2010. An essential role of Nrf2 in American ginseng-mediated anti-oxidative actions in cardiomyocytes. Journal of Ethnopharmacology 130, 222-230.

Li, L., Zhang, J., Sheng, Y., Guo, D., Wang, Q., Guo, H., 2005. Simultaneous quantification of six major active saponins of *Panax notoginseng* by high-performance liquid chromatography-UV method. Journal of Pharmaceutical and Biomedical Analysis 38, 45-51.

Li, T.S.C., Mazza, G., Cottrell, A.C., Gao, L., 1996. Ginsenosides in roots and leaves of American ginseng. Journal of Agricultural and Food Chemistry 44, 717-720.

Li, W., Fitzloff, J.F., 2002. HPLC with evaporative light scattering detection as a tool to distinguish Asian ginseng (*Panax ginseng*) and North American ginseng (*Panax quinquefolius*). Journal of Liquid Chromatography and Related Technologies 25, 17-27.

Li, W., Gu, C., Zhang, H., Awang, D.V.C., Fitzloff, J.F., Fong, H.H.S., Van Breemen, R.B., 2000. Use of high-performance liquid chromatography-tandem mass spectrometry to distinguish *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (North American ginseng). Analytical Chemistry 72, 5417-5422.

Li, W.L., Zheng, H.C., Bukuru, J., De Kimpe, N., 2004. Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. Journal of Ethnopharmacology 92, 1-21.

Ligor, T., Ludwiczuk, A., Wolski, T., Buszewski, B., 2005. Isolation and determination of ginsenosides in American ginseng leaves and root extracts by LC-MS. Analytical and Bioanalytical Chemistry 383, 1098-1105.

Lim, B., Shin, M., Jang, M., Lee, T., Kim, Y., Kim, H., Lee, K., Kim, H., Kim, E., Kim, C., 2002. Ginseng radix increases cell proliferation in dentate gyrus of rats with streptozotocin-induced diabetes. Biological and Pharmaceutical Bulletin 25, 1550-1554.

Lim, J., Wen, T., Matsuda, S., Tanaka, J., Maeda, N., Peng, H., Aburaya, J., Ishihara, K., Sakanaka, M., 1997. Protection of ischemic hippocampal neurons by ginsenoside Rb1, a main ingredient of ginseng root. Neuroscience Research 28, 191-200.

Lim, W., Mudge, K.W., Vermeylen, F., 2005. Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (*Panax quinquefolium*). Journal of Agricultural and Food Chemistry 53, 8498-8505.

Liu, Y., Zhang, J., Li, W., Ma, H., Sun, J., Deng, M., Yang, L., 2006. Ginsenoside metabolites, rather than naturally occurring ginsenosides, lead to inhibition of human cytochrome P450 enzymes. Toxicological Sciences 91, 356-364.

Liu, Z., Zhao, M., Zhang, Y., Xue, J., Chen, N., 2010. Ginsenoside Rg1 promotes glutamate release via a calcium/calmodulin-dependent protein kinase II-dependent signaling pathway. Brain Research 1333, 1-8.

Luu, A., Foster, B.C., McIntyre, K.L., Tam, T.W., Arnason, J.T., 2010. Pharmacogenetics in potential herb-drug interactions: effects of ginseng of CYP3A4 and CYP2C9 allelic variants. Recent Advances in Phytochemistry, in press.

MacKinnon, A., Kershaw, L., Arnason, J.T., Owen, P., Karst, A., Hamersley-Chambers, F., 2009. Edible and Medicinal Plants of Canada. Lone Pine Publishing, Edmonton, Alberta.

Matsuda, H., Samukawa, K., Kubo, M., 1991. Anti-hepatitic activity of ginsenoside Ro. Planta Medica 57, 523-526.

Matsuda, H., Samukawa, K., Kubo, M., 1990. Anty-inflammaotory activity of ginsenoside ro[1]. Planta Medica 56, 19-23.

McIntyre, K.L., Luu, A., Sun, C., Brown, D., Lui, E.M.K., Arnason, J.T., 2010. Ginsenoside variation within and between Ontario ginseng land races: relating phytochemistry to biological activity. Recent Advances in Phytochemistry, in press.

McIntyre, K.L., Harris, C.S., Saleem, A., Beaulieu, L.P., Ta, C.A., Haddad, P.S., Arnason, J.T., 2009. Seasonal phytochemical variation of anti-glycation principles in lowbush blueberry (*Vaccinium angustifolium*). Planta Medica 75, 286-292.

Mihalov, J.J., Marderosian, A.D., Pierce, J.C., 2000. DNA identification of commercial ginseng samples. Journal of Agricultural and Food Chemistry 48, 3744-3752.

Miller, L.G., 1998. Selected clinical considerations focusing on known or potential drug-herb interactions. Archives of Internal Medicine 158, 2200-2211.

Mooney, E.H., McGraw, J.B., 2007. Effects of self-pollination and outcrossing with cultivated plants in small natural populations of american ginseng, *Panax quinquefolius* (Araliaceae). American Journal of Botany 94, 1677-1687.

Naval, M.V., Gómez-Serranillos, M.P., Carretero, M.E., Villar, A.M., 2007. Neuroprotective effect of a ginseng (*Panax ginseng*) root extract on astrocytes primary culture. Journal of Ethnopharmacology 112, 262-270.

Ontario Ginseng Growers Association, 2009. Ginseng Ontario. <http://www.ginsengontario.com >

Predy, G.N., Goel, V., Lovlin, R.E., Basu, T.K., 2006. Immune modulating effects of daily supplementation of COLD-fX (a proprietary extract of North American ginseng) in healthy adults. Journal of Clinical Biochemistry and Nutrition 39, 162-167.

Proctor, J.T.A., Dorais, M., Bleiholder, H., Willis, A., Hack, H., Meier, V., 2003. Phenological growth stages of North American ginseng (*Panax quinquefolius*). Annals of Applied Biology 143, 311-317.

Qiu, Y., Dou, D., Cai, L., Jiang, H., Kang, T., Yang, B., Kuang, H., Li, M.Z., 2009. Dammarane-type saponins from *Panax quinquefolium* and their inhibition activity on human breast cancer MCF-7 cells. Fitoterapia 80, 219-222.

Qu, C., Bai, Y., Jin, X., Wang, Y., Zhang, K., You, J., Zhang, H., 2009. Study on ginsenosides in different parts and ages of Panax quinquefolius L. Food Chemistry 115, 340-346.

Radad, K., Gille, G., Moldzio, R., Saito, H., Rausch, W.-., 2004. Ginsenosides Rb1 and Rg1 effects on mesencephalic dopaminergic cells stressed with glutamate. Brain Research 1021, 41-53.

Rizzi, G.P., 2003. Free Radicals in the Maillard Reaction. Food Reviews International 19, 375-395.

Rosado, M.F., 2003. Thrombosis of a prosthetic aortic valve disclosing a hazardous interaction between warfarin and a commercial ginseng product. Cardiology 99, 111.

Roy, R.C., Grohs, R., Reeleder, R.D., 2003. A method for the classification by shape of dried roots of ginseng (*Panax quinquefolius* L.). Canadian Journal of Plant Science 83, 955-958.

Rudakewich, M., Ba, F., Benishin, C.G., 2001. Neurotrophic and neuroprotective actions of ginsenosides Rb1and Rg1. Planta Medica 67, 533-537.

Scaglione, F., Cattaneo, G., Alessandria, M., Cogo, R., 1996. Efficacy and safety of the standardized ginseng extract G115 for potentiating vaccination against common cold and/or influenza syndrome. Drugs under Experimental and Clinical Research 22, 65-72.

Schlag, E.M., McIntosh, M.S., 2006. Ginsenoside content and variation among and within American ginseng (*Panax quinquefolius* L.) populations. Phytochemistry 67, 1510-1519.

Schripsema, J., 2010. Application of NMR in plant metabolomics: Techniques, problems and prospects. Phytochemical Analysis 21, 14-21.

Schulter, C., Punja, Z.K., 2002. Genetic variation among natural and cultivated field populations and seed lots of American ginseng (*Panax quinquefolius* L.) in Canada. International Journal of Plant Science 163, 427-439.

Sengupta, S., Toh, S., Sellers, L.A., Skepper, J.N., Koolwijk, P., Leung, H.W., Yeung, H.-., Wong, R.N.S., Sasisekharan, R., Fan, T. D., 2004. Modulating angiogenesis: The yin and the yang in ginseng. Circulation 110, 1219-1225.

Shader, R.I. and Greenblatt, D.J., 1985. Phenelzine and the dream machine – ramblings and reflections. Journal of Clinical Psychopharmacology 5, 65.

Shader, R.I. and Greenblatt, D.J., 1988. Bees, ginseng and MAOIs revisited. Journal of Clinical Psychopharmacology 8, 235.

Shi, Y., Huang, T., Chen, L., Pan, X., Zhang, J., Zhu, Y., Chen, X., 2010. Ginsenoside Rg1 attenuates amyloid-β content, regulates PKA/CREB activity, and improves cognitive performance in SAMP8 mice. Journal of Alzheimer's Disease 19, 977-989.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., Guengerich, F.P., 1994. Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. Journal of Pharmacology and Experimental Therapeutics 270, 414-423.

Sievenpiper, J.L., Arnason, J.T., Leiter, L.A., Vuksan, V., 2004. Decreasing, null and increasing effects of eight popular types of ginseng on acute postprandial glycemic indices in healthy humans: The role of ginsenosides. Journal of the American College of Nutrition 23, 248-258.

Sievenpiper, J.L., Arnason, J.T., Leiter, L.A., Vuksan, V., 2003. Variable effects of American ginseng: A batch of American ginseng (*Panax quinquefolius* L.) with a depressed ginsenoside profile does not affect postprandial glycemia. European Journal of Clinical Nutrition 57, 243-248.

Sievenpiper, J.L., Sung, M.K., Buono, M.D., Seung-Lee, K., Nam, K.Y., Arnason, J.T., Leiter, L.A., Vuksan, V., 2006. Korean red ginseng rootlets decrease acute postprandial glycemia: results from sequential preparation- and dose-finding studies. Journal of the American College of Nutrition 25, 100-107.

Singh, S., Singh, R.P., 2008. In vitro methods of assay of antioxidants: An overview. Food Reviews International 24, 392-415.

Singleton, V.L., Rossi Jr, J.A., 1964. Colorimetry of total phenolics with phosphomolbdic-phosphotungistic acid reagents. American Journal of Enology and Viticulture 16, 144-158.

Sloley, B.D., Lin, Y.J., Ridgway, D., Semple, H.A., Tam, Y.K., Coutts, R.T., Löbenberg, R., Tam-Zaman, N., 2006. A method for the analysis of ginsenosides, malonyl ginsenosides, and hydrolyzed ginsenosides using high-performance liquid chromatography with ultraviolet and

positive mode electrospray ionization mass spectrometric detection. Journal of AOAC International 89, 16-21.

Small, E., Catling, P.M., 2003. Canadian Medicinal Plant Biodiversity Priority Issues., in: Arnason, J.T., Catling, P.M., Small, E., Dang, P.T., Lau, A.J. (Eds.), Biodiversity and Health: Focusing Research to Policy. NRC Research Press, Ottawa, pp. 40-54.

Smit, A.J., Lutgers, H.L., 2004. The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. Curr. Med. Chem. 11, 2767-2784.

Tomoda, M., Hirabayashi, K., Shimizu, N., Gonda, R., Ohara, N., Takada, K., 1993. Characterization of two novel polysaccharides having immunological activities from the root of Panax ginseng. Biological and Pharmaceutical Bulletin 16, 1087-1090.

Ulrich, P., Cerami, A., 2001. Protein glycation, diabetes, and aging. Recent Progress in Hormone Research 56, 1-21.

United States Department of Agriculture, 2009. Plants Profile for *Panax quinquefolius* L. American ginseng. <http://plants.usda.gov/java/profile?symbol=PAQU >

Vuksan, V., Sievenpiper, J.L., Wong, J., Xu, Z., Beljan-Zdravkovic, U., Arnason, J.T., Assinewe, V., Stavro, M.P., Jenkins, A.L., Leiter, L.A., Francis, T., 2001. American ginseng (*Panax quinquefolius* L.) attenuates postprandial glycemia in a time-dependent but not dose-dependent manner in healthy individuals. American Journal of Clinical Nutrition 73, 753-758.

Vuksan, V., Stavro, M.P., Sievenpiper, J.L., Beljan-Zdravkovic, U., Leiter, L.A., Josse, R.G., Xu, Z., 2000a. Similar postprandial glycemic reductions with escalation of dose and administration time of American ginseng in type 2 diabetes. Diabetes Care 23, 1221-1226.

Vuksan, V., Stavro, M.P., Sievenpiper, J.L., Koo, V.Y.Y., Wong, E., Beljan-Zdravkovic, U., Francis, T., Jenkins, A.L., Leite, L.A., Josse, R.G., Xu, Z., 2000b. American ginseng improves glycemia in individuals with normal glucose tolerance: Effect of dose and time escalation. Journal of the American College of Nutrition 19, 738-744.

Vuksan, V., Sung, M.K., Sievenpiper, J.L., Stavro, P.M., Jenkins, A.L., Buono, M.D., Lee, K.S., Leiter, L.A., Nam, K.Y., Arnason, J.T., Choi, M., Naeem, A., 2008. Korean red ginseng (Panax ginseng) improves glucose and insulin regulation in well-controlled, type 2 diabetes: results of a randomized, double-blind, placebo-controlled study of efficacy and safety. Nutrition, Metabolism and Cardiovacsular Diseases 18, 46-56.

Wan, J.-., Li, S.-., Chen, J.-., Wang, Y.-., 2007. Chemical characteristics of three medicinal plants of the *Panax* genus determined by HPLC-ELSD. Journal of Separation Science 30, 825-832.

Wang, A., Wang, C.-., Wu, J.-., Osinski, J., Yuan, C.-., 2005. Determination of major ginsenosides in *Panax quinquefolius* (American ginseng) using high-performance liquid chromatography. Phytochemical Analysis 16, 272-277.

Wang, X., Sakuma, T., Asafu-Adjaye, E., Shlu, G.K., 1999. Determination of ginsenosides in plant extracts from *Panax ginseng* and *Panax quinquefolius* L. by LC/MS/MS. Analytical Chemistry 71, 1579-1584.

Wang, Y.-., Du, G.-., 2009. Ginsenoside Rg1 inhibits β-secretase activity in vitro and protects against Aβ-induced cytotoxicity in PC12 cells. Journal of Asian Natural Products Research 11, 604-612.

Wen, H., Kang, S., Song, Y., Song, Y., Sung, S.H., Park, S., 2010. Differentiation of cultivation sources of *Ganoderma lucidum* by NMR-based metabolomics approach. Phytochemical Analysis 21, 73-79.

World Health Organization, 2009. Diabetes Programme- Facts and Figures. <http://www.who.int/diabetes/en/>

Yim, T.K., Ko, K.M., Ko, R., 2002. Antioxidant and immunomodulatory activities of Chinese tonifying herbs. Pharmaceutical Biology 40, 329-335.

Yu, L., Zhang, X., Li, S., Liu, X., Sun, L., Liu, H., Iteku, J., Zhou, Y., Tai, G., 2010. Rhamnogalacturonan I domains from ginseng pectin. Carbohydrate Polymers 79, 811-817.

Yuan, C.S., Wei, G., Dey, L., Karrison, T., Nahlik, L., Maleckar, S., Kasza, K., Ang-Lee, M., Moss, J., 2004. Brief communication: American ginseng reduces warfarin's effect in healthy patients. Annals of Internal Medicine 141, 23-28.

Zanger, U.M., Turpeinen, M., Klein, K., Schwab, M., 2008. Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. Analytical and Bioanalytical Chemistry 392, 1093-1108.

Zhang, X., Yu, L., Bi, H., Li, X., Ni, W., Han, H., Li, N., Wang, B., Zhou, Y., Tai, G., 2009. Total fractionation and characterization of the water-soluble polysaccharides isolated from Panax ginseng C. A. Meyer. Carbohydrate Polymers 77, 544-552.

Zhang, Y., Zhang, H., Zhang, G., Fan, J., Li, X., Liu, Y., Li, S., Lian, X., Tang, Z., 2008. Panax notoginseng saponins attenuate atherosclerosis in rats by regulating the blood lipid profile and an anti-inflammatory action. Clinical and Experimental Pharmacology and Physiology 35, 1238-1244.

Zhou, S., Brown, D.C.W., 2006. High efficiency plant production of North American ginseng via somatic embryogenesis from cotyledon explants. Plant Cell Reports 25, 166-173.

Zhou, S.F., Xue, C.C., Yu, X.Q., Li, C., Wang, G., 2007. Clinically important drug interactions potentially involving mechanism-based inhibition of cytochrome P450 3A4 and the role of therapeutic drug monitoring. Therapeutic Drug Monitoring 29, 687-710.

Zhu, Y., Pettolino, F., Mau, S., Bacic, A., 2005. Characterization of cell wall polysaccharides from the medicinal plant *Panax notoginseng*. Phytochemistry 66, 1067-1076.

Zhu, S., Zou, K., Cai, S., Meselhy, M.R., Komatsu, K., 2004. Simultaneous determination of triterpene saponins in ginseng drugs by high-performance liquid chromatography. Chemical and Pharmaceutical Bulletin 52, 995-998.