INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Université d'Ottawa · University of Ottawa

PHYTOCHEMICAL VARIATION AND IMMUNOPHARMACOLOGY OF *PANAX QUINQUEFOLIUS* L. (AMERICAN GINSENG)

A thesis submitted to the
Faculty of Graduate and Postdoctoral Studies

in partial fulfilment of the requirements for the degree of
Doctor in Philosophy

in the Ottawa-Carleton Institute of Biology
Department of Biology
University of Ottawa
Ottawa, Ontario
Canada

Candidate

Valerie Ann Assinewe

Supervisor

Professor John Thor Arnason

Valerie Ann Assinewe, Ottawa, Ontario Canada –December 2001

DEDICATION

This study is dedicated to Mary Begoniasing, Charlotte Toulouse, and the other grandmothers who have carried on the tradition of medicinal use of local plants.

ABSTRACT

The ethnopharmacological use of *Panax quinquefolius* L. (American ginseng) led to assessment of the immunostimulating properties of this eastern North American herb, and to investigate the phytochemical variation in its associated North American Araliaceae relatives. An analysis of the effect of different processing methods on ginsenoside content, a determination of the bioactive compounds in ginseng, and testing of an HPLC method were also undertaken in this thesis.

A survey of wild North American *P. quinquefolius* populations showed phytochemical variation exemplified by differences in the levels of major ginsenosides between populations. In this study age was not a factor in this variation, and there was no statistical difference in ginsenoside content between wild and cultivated *P. quinquefolius* roots. Baseline data on total ginsenoside levels for authentic *P. quinquefolius* was produced; this will be a resource to forest ginseng growers.

The investigation of the phytochemical variation in eastern North American Araliaceae revealed the presence of ginsenosides in *Aralia nudicaulis* L. (Wild sarsaparilla) and *Aralia racemosa* L. (Spikenard). Major ginsenosides were also quantified in *P. quinquefolius* stem, and the ginsenoside-R_{b2} was shown to be a significant saponin in *P. quinquefolius* leaves. Several ginsenosides were identified and quantified in *Panax trifolius* L. (Dwarf ginseng): the ginsenosides R_{g1} and R_e in the leaves, R_{g1} and R_c in the stem, and R_d in the roots. Compared to *P. quinquefolius,* in which the highest total ginsenosides were in the root, ginsenosides occurred primarily in the aerial parts in the other three Araliaceae, and the compounds were at much lower

levels. *P. trifolius* leaves, however, contained a level of ginsenoside comparable to that of *P. quinquefolius* leaves.

Two investigations of the effects of different methods of ginsenoside analysis were undertaken as part of this thesis. The AOAC method of validation for ginseng analysis was an international collaborative study. The goal was to validate an HPLC method for the determination of the major ginsenosides R_{b1}, R_{b2}, R_c, R_d, R_e, and R_{g1}, and to use it to estimate the ginsenoside content in four ginseng samples. Although the results obtained for system suitability, intra-laboratory repeatability, and inter-laboratory reproducibility were generally consistent among the collaborators, the HPLC method was held not to be rigorous enough for the analysis of R_{g1} and R_e. The second investigation of ginsenoside analyses examined the effect of different processing methods. The results for this investigation indicated that the most promising processing method for obtaining the highest levels of total ginsenoside content and the best quantities of R_{b1}, R_{g1} and R_d was drying at high temperature, as performed to obtain white ginseng. A pre-steam treatment before drying to obtain red ginseng and particularly freeze-drying yielded lower ginsenoside levels.

Some immunostimulating properties of *P. quinquefolius* were assessed. Aqueous extracts from a four-year old cultivated root significantly stimulated the release of immunoreactive tumour necrosis factor alpha (TNFα) in culture. A ginsenoside-rich fraction obtained from a methanol extraction of the root showed little activity, as did the pure ginsenoside-R_{b1}. However, the soluble polysaccharide-enriched fraction, which was a water extract of the insoluble residue from the methanol extraction, stimulated TNFα release to the same extent as the total root extract.

The crude extractable polysaccharide fraction was analysed, and was found to contain glucose, uronic acid, galactose, arabinose, rhamnose, fucose and mannose.

RÉSUMÉ

L'utilisation ethnopharmacologique de *Panax quinquefolius* L. (Ginseng américain) nous a conduit à évaluer les propriétés de cette plante de l'Est nord-américain, ainsi qu'a étudier la variation phytochimique vis-à-vis des Araliacées canadiennes proches. De plus, nous avons entrepris une analyse de l'effet de différents procédés sur le contenu en ginsenoside, la détermination des composes bioactifs du ginseng et le test d'une méthode HPLC.

Un suivi des populations sauvages de *P. quinquefolius* a montré une variation phytochimique causée par des différences dans les niveaux des ginsenosides entre les populations.

L'étude a montré que l'âge n'est pas un facteur impliqué dans cette variation et qu'il n'y a pas de variations significatives dans la composition en ginsenoside entre les racines de *P. quinquefolius* sauvages et cultivés sont observables. Les données de base sur les concentration de ginsenosides pour l'authentique *P. quinquefolius* ont été acquises; ce sera une reference pour les cultivateurs forestiers de Ginseng.

L'investigation de la variation phytochimique chez les Araliacées nord-américaine a révélé la présence de ginsenosides dans *Aralia nudicaulis* L. (sarsaparilla sauvage) et *Aralia racemosa* L., (spikenard). Les ginsenosides majeurs ont été aussi quantifiés dans les tiges de *P. quinquefolius* et une présence significative du ginsenoside-R_{b2} a été montrée dans les feuilles de cette même espèce. Plusieurs ginsenosides ont été identifiés et quantifiés dans *Panax trifolius* L. (ginseng nain): les ginsenosides R_{g1} et R_e dans les feuilles, R_{g1} et R_c dans les tiges, et R_d dans les racines. Par comparaison,

avec *P. quinquefolius*, les ginsenosides des trois autres Araliacées sont majoritairement présents dans les parties aériennes de plus les composés sont en de très faibles quantités. Toutefois, les feuilles de *P. trifolius* contiennent des niveaux de ginsenosides comparables à ceux des feuilles de *P. quinquefolius.*

Deux investigations des effets de différentes méthodes d'analyse des ginsenosides ont été entreprises au cours de cette thèse. La méthode AOAC de validation pour l'analyse du ginseng a été le résultat d'une collaboration internationale. Le but était de valider une méthode HPLC pour la détermination des ginsenosides majeurs R_{b1}, R_{b2}, R_c, R_d, R_e, et R_{g1} et l'utiliser pour déterminer la composition dans 4 échantillons de ginseng. Bien que les résultats obtenus pour la compatibilité du système, la répetition intra-laboratoire et la reproductibilité inter-laboratoire étaient cohérents entre les collaborateurs, la méthode HPLC n'a pas été considérée comme assez rigoureuse pour l'analyse de R_{g1} et R_e. La seconde étude a examiné l'effet de différentes méthodes de préparation. Les résultats indiquent que la méthode la plus prometteuse pour l'obtention de teneurs en ginsenosides les plus élevées et les meilleures quantités en R_{b1}, R_{g1}, et R_d est le séchage a haute température, comme utilisé pour le Ginseng blanc. Une pré-vaporisation pour obtenir le ginseng rouge et particulièrement la lyophilisation diminuent les niveaux en ginsenoside.

Les propriétés immunostimulantes de *P. quinquefolius* ont été évaluées. Les extraits aqueux de racines cultivées depuis 4 ans stimulent de façon significative la libération de l'immunoreactif TNFα dans le surnageant de culture. Une fraction riche en ginsenoside obtenu à partir d'un extrait de racine au méthanol montrent peu d'activité, comme le ginsenoside R_{b1} pur. Toutefois, la fraction soluble enrichie en

polysaccharides, extrait aqueux du résidu insoluble de l'extraction au méthanol, stimule la production de TNFα dans les même proportions que l'extrait racinaire total.

La fraction crue des polysaccharides extractrable a été analysée et contient du glucose, acide uronique, galactose, arabinose, rhamnose, fucose et mannose.

ACKNOWLEDGEMENTS

I would first like to acknowledge the financial aid in grants, tuition fees and incentive awards that I received from the Sudbury Area Post Secondary Counselling Unit, and the much-appreciated support of Marvin Assinewai.

I am grateful to Don Cuddy of Ontario Natural Resources who donated roots to me and was instrumental in helping me obtain a permit to collect *Panax trifolius* and *Aralia* spp. in Frontenac Park.

Thanks to everyone who went on collecting trips with me, especially Dr. J. Thor Arnason, Bryan Assinewe, Fern Assinewe, Laura Assinewe, Linda Marie Assinewe, Maani Assinewe, Martin Assinewe, Ann-Sophie Belzile, Shannon Binns, Leslie Bristo, Jeff Bullard, Peter Fang, Dr. Daniel Gagnon, George Hargrave, Dr. Robin Marles, Luc St. Laurent, Larry Osgood, Nick Stow, Terry Rudden, and David White.

Sharon Cuddy of Sault College, Madeleine Gaboury, John Race of HBR Ginseng Ltd, Luc St. Laurent, and David Sharkey all helped me tremendously by donating plant parts.

I received very valuable information on ginsenoside content from Dr. Dennis Awang of MediPlant, Dr. Jim Brandle of Agriculture and Agri- Food Canada, and Tara Hall of the American Botanical Council.

Dr. Patrick Yat was of great assistance in helping me set up my procedures for extractions and HPLC.

I am grateful to Dr. Irma Lemaire for allowing my to work in her lab. My research there has given me great appreciation for the advances in science that have been

achieved in pharmacology. I thank Annie Aubry at Dr. Irma Lemaire's laboratory, whose patience, help and good cheer was unflagging as we worked through the immunopharmacological part of this study.

Dr. W. John Mullin of Agriculture and Agri-food Canada carried out the polysaccharide analyses. Shannon Binns provided much appreciated assistance in the canonical analyses.

I thank the members of my Advisory Committee who guided my research and gave me assistance and encouragement: Dr. Irma Lemaire, Dr. Frances Pick, Dr. Daniel Gagnon and Dr. Myron Smith. I am especially grateful for the support of Professor J. Thor Arnason, my thesis supervisor, who understood the purpose of my study, provided invaluable advice at several key points in my research, and was always there when I required help. It was very fortunate for us both that he understood my sense of humour.

Finally, I want to recognize Terry Rudden, my husband, who for the past decade has supported me financially, intellectually, and emotionally.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

GENERAL INTRODUCTION: BACKGROUND, BOTANY, PHYTOCHEMISTRY AND PHARMACOLOGY OF *PANAX QUINQUEFOLIUS* L. (AMERICAN GINSENG)

1.1 Introduction

In his memoirs published in Paris (Trévoux, 1717, 1718), the Jesuit missionary Joseph Francois Lafitau (1668 – 1720) recalled that in 1715, during the early days of his mission at Sault Saint-Louis (now Caughnawaga, near Montreal QC), he first learned of ginseng through the letters of a colleague, Father Petrus Jartoux (1681 – 1746). Writing of his 1709 geographic survey of Tartary (Manchuria and China) for the Chinese emperor, Father Jartoux described a plant highly prized in Chinese traditional medicine; one he believed could be found in similar temperate terrain. Father Lafitau eventually located the plant close to his home at the mission in 1716. A Mohawk woman, who worked for the mission, described the plant to him as an ordinary medicine used to treat fevers, pleurisy, flux of the blood and tuberculosis, and as a general restorative and tonic.

Father Lafitau is often credited as the discoverer of American ginseng, and in fact there is doubt by some that the plant was used by the First Nations peoples prior to Lafitau's "discovery" (Carlson, 1986; Hardacre, 1974). Father Lafitau's memoirs, however, clearly show that Aboriginal people already had knowledge of this plant. While he may not have "discovered" American ginseng, Lafitau should nevertheless

be credited with initiating the considerable and often lucrative 280-year export of roots to the Orient that resulted from his identification of this plant.

1.1.1 Panax quinquefolius L. (American ginseng)

The Araliaceae (ginseng family) is an important plant family worldwide. It includes many species used in traditional medicines, as well as species used in mass-market phytopharmaceuticals. The Chinese have used *Panax ginseng* C.A. Meyer (Asiatic ginseng) for at least 5,000 years (Tanaka 1990). The antiquity of use of *Panax quinquefolius* L. (American ginseng), a close relative of *P. ginseng* (Li, 1952 and 1972), and *Aralia* spp. by Aboriginal peoples of North America is well established in oral tradition and historical reports (Moerman, 1998; Arnason et al., 1981; Erichsen-Brown, 1979).

Despite isolation of many of the plant's constituents, the pharmacology of *P. quinquefolius* and related family members is still relatively unknown. In this thesis, I characterize the phytochemical variation within the *P. quinquefolius* gene pool and related species, and assess some of the pharmacological properties of *P. quinquefolius*.

1.1.2 Literature Review

The Harlan and deWet (1971) system can be used to describe the *P. quinquefolius* germplasm resources and its closest relatives (Figure 1.1). All populations, varieties and landraces of *P. quinquefolius* are designated as "GP-1"

Figure 1.1 Germplasm resources of ginseng (modified from Wielgorskaya, 1995; Gleason, 1968).


GP-3
In northeastern North America
Aralia hispida
Aralia nudicaulis
Aralia racemosa
Aralia spinosa
Oplopanax horridus
50 species of *Aralia* and 3 species of *Oplopanax*
are found worldwide.
GP-2
In North America
Panax trifolius
10 other *Panax* species worldwide.
GP-1
Panax quinquefolius

where hybridization is easy and hybrids are fertile. "GP-2" includes close relatives where hybridization is possible but difficult. An example of GP-2 is a hybrid of *P. ginseng* and *P. quinquefolius.* The hybrid itself has proven to be sterile, but hairy root cultures from the hybrid have produced greater amounts of ginsenosides than either of the parental species (Washida et al., 1998). "GP-3" describes populations where hybridization is unlikely to occur naturally but where the species are related.

1.1.3 Traditional uses

Araliaceous plants have traditionally been used as medicines. *P. ginseng*, the best-known member of this family, has a long history of medicinal use by East Asians, and is used in both the East and the West to promote good health by stimulating the metabolism, and as a tonic during convalescence (Li, 1995; Raman and Houghton, 1995; Fulder, 1980). The Aboriginal peoples of North America used American ginseng for its healing properties long before French and English colonization (Pritts, 1995).

Traditionally, the Iroquois used *P. quinquefolius* root infusions to treat fever; the Maritime Indians used the root as a blood detergent; and the Ojibwa used the root to alleviate stomach pain (Arnason et al., 1981). Foster (1996) recorded that other Aboriginal peoples of North America used the root to relieve childbirth pain, cure nosebleed, treat shortness of breath, strengthen mental powers, increase the fertility of women, and as a general tonic.

P. quinquefolius, considered more 'yin' (cool) in Traditional Chinese Medicine, is marketed for its ability to 'cool' the respiratory and digestive systems, and *P.*

ginseng, considered more 'yang' (warm), is marketed for its heating effect on the circulatory system (Foster, 1996).

1.1.4 Nomenclature

In 1753, Carolus Linnaeus (1707 – 1778), who developed the binomial system of biological classification, christened American ginseng as *Panax quinquefolium*. The genus name reflects the ethnopharmacological use of the herb as a remedy for all diseases: in Greek *pan* means "all" and *akos* means "a remedy" (Quattrocchi, 2000; Linneau, 1753, 1754). The species epithet refers to the plant's five leaflets. It is notable that *P. ginseng*, the most valued of the Asian species of Araliaceae, was not officially described until almost 100 years later.

Article 62.1 of the International Code of Botanical Nomenclature (Greuter et al., 2000) indicates that a generic name retains the gender assigned by botanical tradition, irrespective of classical usage or the author's original usage—in this case Linnaeus'. Consequently, by this authority, the correct binomial name for American ginseng is *Panax quinquefolius*.

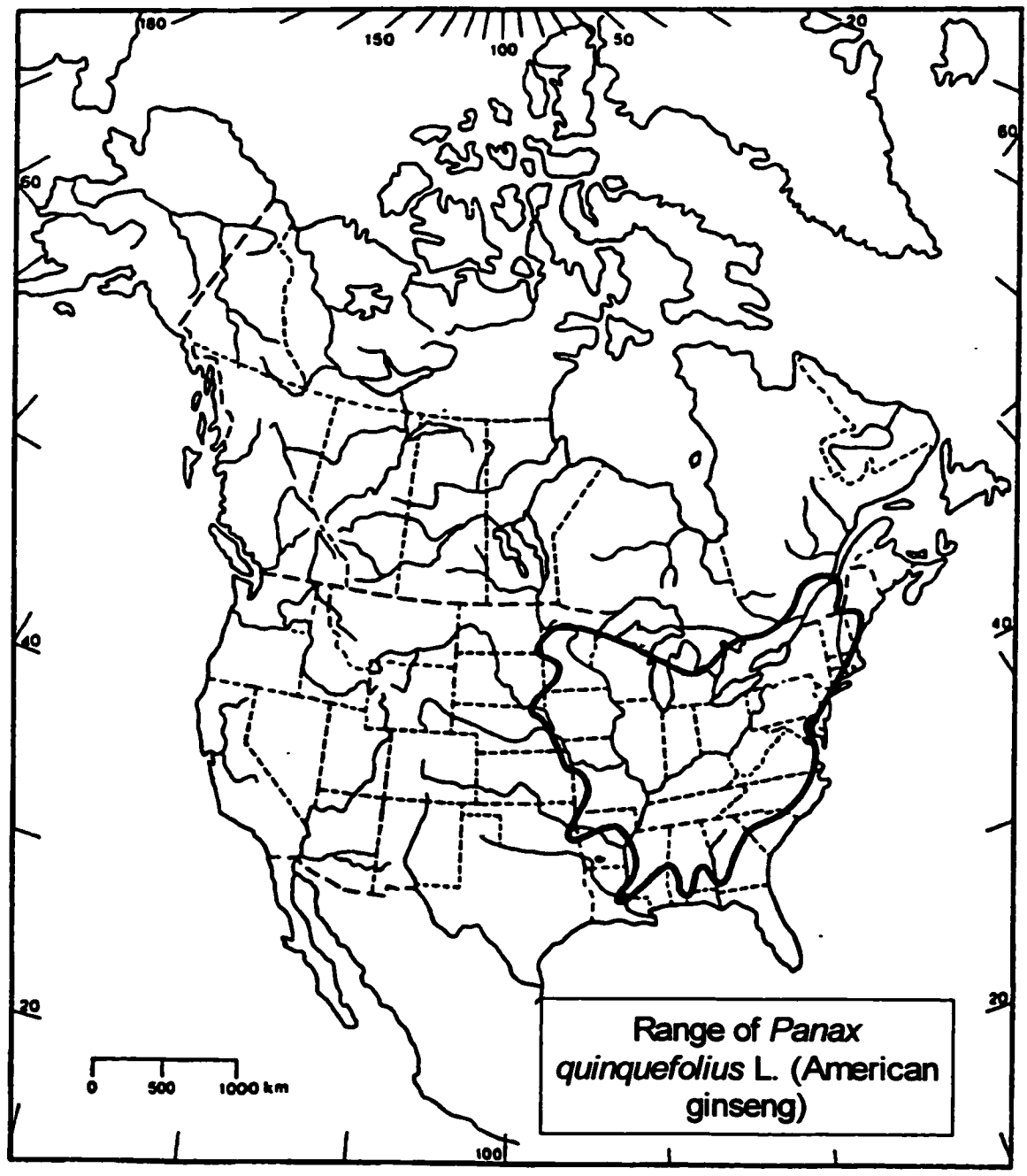
Some common names for *P. quinquefolius* are American ginseng, North American ginseng, Canadian ginseng, sang, and seng. The last two names are derived from the Chinese 'ren-shen', which has been translated as 'man-shaped root' (Small and Catling, 1999).

1.1.5 Status and Ecology

P. quinquefolius is an herbaceous perennial endemic to North America. It was once prolific in the under story of eastern North American mixed deciduous forest (Charron and Gagnon, 1991; Lewis and Zenger, 1982). This forest is dominated by *Acer saccharum* Marsh., Aceraceae (Sugar maple), and is generally mature with a closed canopy, sparse shrub under story, and diverse ground vegetation (White, 1988). *P. quinquefolius* grows on the northern, northwestern, or northeastern slope at mid- to low-slope positions in rich, damp but well-drained and undisturbed soil often associated with limestone and marble bedrock (White, 1988). The natural range extends from its northern limit in southern Quebec and Minnesota to its southern limit in northern Florida to Louisiana (Figure 1.2).

In 1999, *P. quinquefolius* was reassessed and was up-listed from the 'threatened' designation of 1988 (White, 1988) to 'endangered' in Ontario and Quebec by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (Nault and White, 1999). COSEWIC determines the national status of wild species that are considered to be at risk in Canada. In the United States of America, there is concern about the long-term survival of the species in 22 of the 35 states that are part of *P. quinquefolius*' natural range (Robbins, 1998). In both countries the endangerment of the species is due to the continued over-harvest of the natural supply of ginseng roots and the loss of hardwood forests due to human activity.

Figure 1.2 Natural range of *Panax quinquefolius* L. (American ginseng) (Argus et al., 1984).


180
150
100
50
20
60
60
40
40
20
20
100
0
500
1000 km
Range of *Panax quinquefolius* L. (American ginseng)

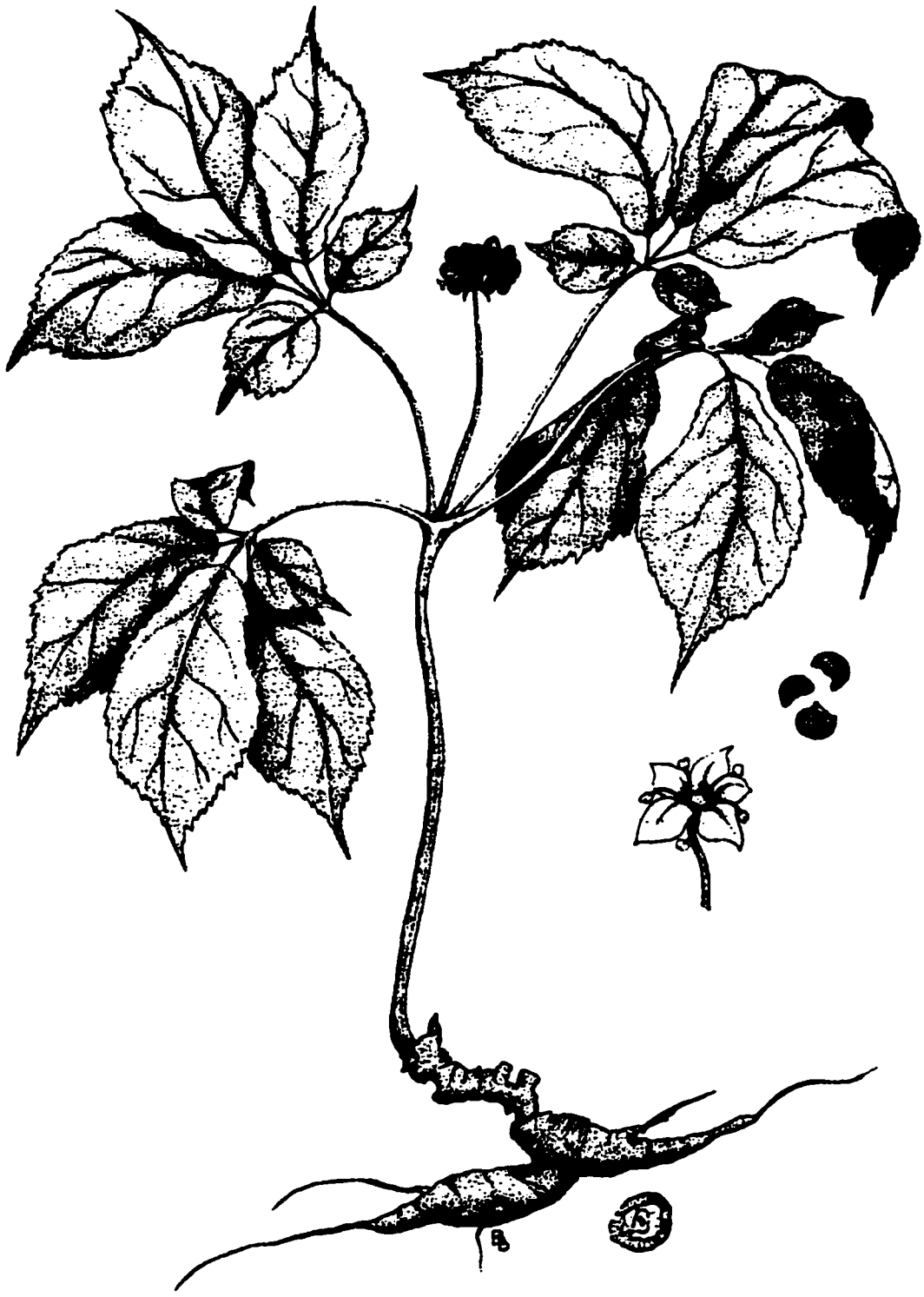
1.1.6 Morphology

P. quinquefolius has an underground rhizome bearing scars of the yearly loss of the stem, which are used to age the plant (Carpenter and Cottam, 1982), and a fleshy root 5 – 10 cm long, similar in shape to a carrot, often forked with root hairs and acquiring prominent circular wrinkles as it ages; the root is aromatic and has a slightly bitter taste. The aerial portion of the plant is 20 – 70 cm tall, with a single annual erect stem bearing a terminal verticil or whorl of long-stalked palmately compound leaves. Each leaf has five large leaflets, of which the upper three are larger than the lower two. Seedlings initially have 3 leaflets; in subsequent years the plants develop 3 - 5 compound leaves each with 3 – 7, normally 5 leaflets (Thompson, 1987). In some literature the leaves are referred to as prongs (Lewis and Zenger, 1982). Self-pollination occurs more than cross pollination in the 4 week period in mid-summer once the 3+ year old plants have produced 6 – 20 small, greenish-white, pentamerous flowers borne on a terminal simple umbel on a short stalk arising from the center of leaf attachment at the top of the stem (Schluter and Punja, 2000, Lewis and Zenger, 1982). Pea-sized fruits developed from inferior ovaries ripen at the end of July. Although the bright red berries would suggest animal dispersal, most fruits are passively disseminated within a meter of the plant (White, 1988). (Figure 1.3)

1.1.7 Agricultural and Commercial Aspects

Canadian export of wild American ginseng to China began in 1721, following Father Lafitau's first documentation of the plant's existence: its export value was

Figure 1.3 Illustration of *Panax quinquefolius* L. (American ginseng) (Small and Catling, 1999).



second only to fur (Evans, 1985). The Chinese have since prized this herb for its healing and health promoting properties (Duke, 1989; Proctor et al., 1988).

Over time the wild ginseng population became severely depleted as a result of over-harvesting. Commercial cultivation of American ginseng in Canada began in the late 1890's, and experienced exponential growth in the 1930's (Fisher, 1993). Statistics Canada (1999) reported that by 1998, ginseng production in Canada had reached more than 1800 metric tonnes compared to less than 25 in 1982; more than 2000 hectares were involved in ginseng production (~ 1/3 in British Columbia and ~ 2/3 in Ontario), and the plants' export value increased from ~ $3 million Can in 1982 to ~ $61 million Can in 1998. Seventy-five percent of Canadian ginseng production is exported annually (Small, 1997). Shaw and But (1995) reported that *P. quinquefolius* is sold for prices five to ten times higher than *P. ginseng* in the Hong Kong market, the principal clearinghouse for trade in ginseng. In the North American mainstream market, ginseng ranks in the top three of the commercial herbal products in retail sales in each of the last five years (Hall et al., 2001; Brevoort, 1996).

Cultivated ginseng is the primary source of ginseng products sold today (Robbins, 1998). *P. quinquefolius* is also profitably cultivated in China (Proctor et al., 1988), and attempts are underway to establish ginseng production in other countries, i.e. New Zealand (Smallfield et al., 1995).

1.2 Phytochemical Constituents of Ginseng

1.2.1 Ginsenosides

The main class of bioactive compounds in ginseng has been shown to be dammarane-type saponins (tetracyclic triterpenoid glycosides), commonly referred to as ginsenosides, which are its major secondary metabolite constituents (Tanaka, 1990; Shibata et al., 1985). The arrangement of sugar residues: glucose, rhamnose, xylose and arabinose, are shown as R^1, R^2 and R^3 in Figure 1.4 as well as some basic molecular information. Although there are now more than 30 reported ginsenosides (Smith et al, 1996), the most abundant ginsenosides in the *Panax* plants are R_{b1}, R_{b2}, R_c, and R_d, which have a (20*S*)-protopanaxadiol aglycone, and R_e, R_f, R_{g1}, which have a (20*S*)-protopanaxatriol aglycone (Shibata et al., 1985).

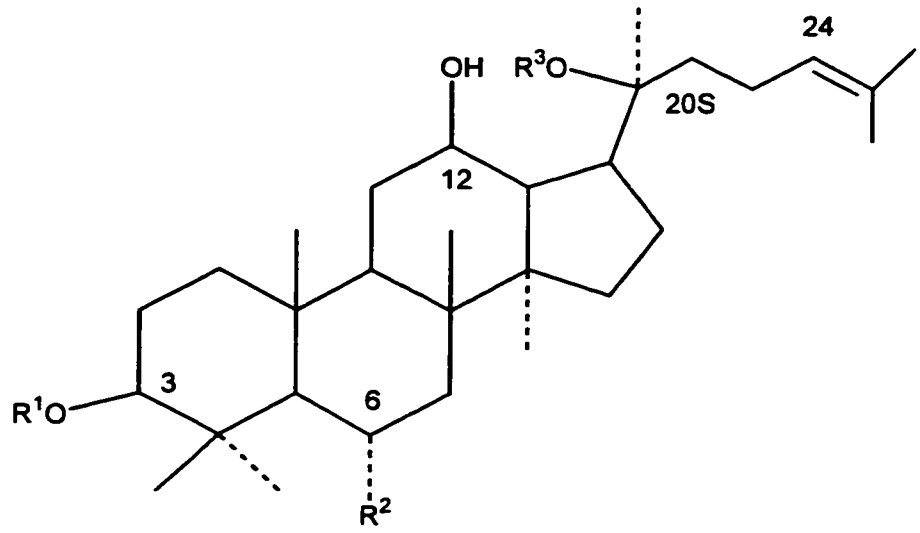
Peterson et al. (1998), in an anatomical study of the *P. quinquefolius* root, localized ginsenosides to schizogenous ducts.

Other dammarane-type triterpenes specific to *P. quinquefolius* are the oligoglycosides quinquenosides I, II, III, IV, and V (Yoshikawa et al., 1998).

1.2.1.1 Factors Affecting Ginsenoside Concentration

Ginsenosides concentrations vary in different parts of the plant. The ginsenoside content in *P. ginseng* decreases in the order bud-flower > leaf > root > rhizome > seed (Nah, 1997; Liu and Xiao, 1992). Various parts of the root also have different ginsenoside content: the main root of a five-six year old *P. ginseng* root normally contains between 0.7 – 3.0 % total saponins (Soldati and Tanaka, 1984);

Figure 1.4 Chemical structures of the seven most common ginsenosides.



Ginsenoside	R^1	R^2	R^3	Molecular formula	Molecular weight
-R_{b1}	Glc-^{2}Glc-	H	Glc-^{6}Glc-	$C_{54}H_{92}O_{23}$	1109
-R_{b2}	Glc-^{2}Glc-	H	Ara(p)-^{6}Glc-	$C_{53}H_{90}O_{22}$	1079
-R_c	Glc-^{2}Glc-	H	Ara(f)-^{6}Glc-	$C_{53}H_{90}O_{22}$	1079
-R_d	Glc-^{2}Glc-	H	Glc-	$C_{48}H_{82}O_{18}$	947
-R_e	H	Rha-^{2}Glc-O-	Glc-	$C_{48}H_{82}O_{18}$	947
-R_f	H	Glc-^{2}Glc-O-	H	$C_{42}H_{72}O_{14}$	801
-R_{g1}	H	Glc-O-	Glc-	$C_{42}H_{72}O_{14}$	801

Glc: β-D-glucopyranosyl, Ara(f): α-L-arabinofuranosyl, Ara(p): α-L-arabinopyranosyl, Rha: α-L-rhamnopyranosyl (Modified from Shibata et al., 1985 and van Breeman et al., 1995)

whereas the adventitious roots have three times more, while the hair roots may have as much as ten times more (Sonnenborn and Proppert, 1991).

P. quinquefolius leaves contain mainly the R_d and R_e ginsenosides, the roots mainly the R_{b1} and R_e ginsenosides; so that as the leaves mature and accumulate these compounds, there is a corresponding decrease in the roots (Li et al., 1996). In comparison to *P. ginseng*, *P. quinquefolius* has more total ginsenoside (Smith et al., 1996; Li, 1995; Soldati and Sticher, 1980). A characteristic ginsenoside in *P. quinquefolius* is (24*R*)-pseudoginsenoside-F_{11}, but it does not have the ginsenoside-R_f (Li et al., 1996; Shibata et al., 1985); consequently the presence or absence of these compounds distinguishes the two species.

Influences from the environment affect the chemical profile of populations. Such influences include soil nutrients and texture, humidity of soil and air, and sun conditions. As a result, there is variation in the concentration of ginsenosides found in plants of the same species. For example, R_{b1}, R_c, and R_d have been shown to be most susceptible to varying environmental conditions (Li et al., 1996). Foster (1996) noted that wild *P. quinquefolius* root has higher levels of ginsenosides than the cultivated root. However, this claim was not verified with statistical analyses.

Genetic variation among populations of the same species also plays a role in the chemical content of the population (Baldwin and Preston, 1999; Bazzaz et al., 1987).

The age of the plant also effects ginsenoside concentrations. Court et al. (1996) showed that ginsenoside concentrations in *P. quinquefolius* root increase with age: this is specifically attributable to R_{b1}, mR_{b1}, R_e and R_o. They recommended

a harvest of the ginseng crop at age 4 rather than 3 years to obtain a higher amount of ginsenosides. Soldati and Tanaka (1984) showed similar results for cultivated *P. ginseng*; moreover, their study determined that there is no marked increase in ginsenoside concentrations after the fifth summer.

Although R_{b1}, R_{b2}, R_{b3}, R_c, R_d, R_e, R_f, and R_{g2} have been found in *P. trifolius*, some of the compounds have not been quantified (Lee and Der Marderosian, 1981, 1988a, 1988b; Lui and Staba, 1980).

Ginsenoside occurrence has not been investigated in the *Aralia* spp., the other major genus in the ginseng family (Figure 1.1).

1.2.2 Polyacetylenes

Polyacetylenes are minor bioactive components of ginseng. They comprise part of the 1% lipid soluble compounds in ginseng (Nah, 1997). All the components are structurally related: they are either C_{17}- or C_{18}-polyacetylenes with a characteristic terminal saturated aliphatic C_7H_{15} moiety for the C_{17}-compounds and a 3-hydroxy(or 3-oxo)hept-1-ene-4,6-diyne moiety for the C_{18}-compounds (Hansen and Boll, 1986). Table 1.1 lists the polyacetylenes investigated in the eastern Canadian members of the ginseng family.

1.2.3 Polysaccharides

Many cell wall-derived polysaccharides have been isolated from *P. ginseng* by different fractionation procedures, including panaxans A to U, and ginsenan PA and ginsenan PB. Of these, only panaxans A to E and the ginsenans have been well

Table 1.1 Polyacetylenes in Araliaceae.

Species	Compounds isolated	References
Aralia nudicaulis	falcarinone	Hansen and Boll, 1986
	falcarinolone	
Aralia racemosa	falcarinolone	Hansen and Boll, 1986
Panax quinquefolius	falcarinol (panaxynol)	Fujimoto et al., 1991
	panaxydol	
	panaxytriol	
	heptadeca-1,8-diene-4,6-diyne-3,10-diol	
	PQ-1	
	PQ-2	
	PQ-3	
	6'-O-acetyl-ginsenoside-R_{g1}	Yoshikawa et al., 1998

characterized (Tomoda et al., 1993a, 1993b). Most of these polysaccharides have high molecular weights, generally in the order of 10 – 100 kDa, and are heteroglycans containing primarily galactose, arabinose and glucose in complex structures of branching chains (Lien and Gao, 1990). These characteristics also describe the polysaccharides quinquefolans A, B, and C isolated from *P. quinquefolius* roots (Oshima et al., 1987).

1.2.4 Other constituents

Other phytochemicals in *P. quinquefolius* include the flavonoids kaempferol-3,7-dirhamnoside and kaempferol-3-gluco-7-rhamnoside (Lee and Der Marderosian, 1988a, 1988b), and the sesquiterpenes α-panasinene, β-panasinsene and β-neoclovene (Iwabuchi et al., 1987).

1.3 Pharmacognosy of constituents

In allopathic medicine, several medicinal and therapeutic effects of ginseng have been established using *in vitro* studies, animal trials and clinical studies. These include the use of *P. ginseng* in such immunostimulating studies as the augmentation of murine lymphocyte proliferation (Mizuno et al., 1994) and natural killer (NK) cell function (Jie et al., 1984), and in human studies the increase of neutrophil function, CD4 cell count and NK cell function (See et al., 1997; Scaglione et al., 1996; Scaglione et al., 1990).

Ginseng root is now classified in alternative medicine as an "adaptogen", a term defined by Wagner et al. (1994) as a plant drug that potentiates non-specific resistance against long-term stress.

1.3.1 Ginsenosides

Natural ginsenosides, R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2} and R_d, possess very low cytotoxicity. Atopkina et al. (1999) showed that aglycones are more toxic than the seven major ginsenosides, and semi-synthetic analogues of the natural ginsenosides having the least number of sugar moieties were more cytotoxic than those analogues with a greater number of sugar moieties. They speculate that since the number of sugar moieties determines the hydrophilic properties of the compound, the greater number of sugar moieties holds the compound in the cell membrane thus lowering its cytotoxicity.

One of the more important pharmacological activities associated with ginseng is its action as an immunomodulator. Singh et al. (1983) were the first to show experimentally the immunostimulating ability of *Panax ginseng*. This led to a study of the effect of ginseng extract on the expression of humoral and cell mediated immune response. In 1984, Sing et al. reported the inhibition in macrophage migration and increase in natural killer cell and interferon activities observed in mice pre-treated with *P. ginseng*. In 1996, Akagawa et al. reported that a root extract of *P. ginseng* enhanced the activity of peritoneal macrophages in C3H/He J mice infected intravenously with the fungus *Candida albicans*. No mechanisms for these activities were reported, although Akagawa et al. speculated that the extract either directly

activated the macrophages, or induced the production of cytokines such as interleukin-2 (IL-2) and interferon-gamma (ILγ) from mononuclear cells, which would then activate other effector cells in anti-*Candida* growth.

Kenarova et al. (1990) reported that ginsenoside R_{g1} modulated both humoral and cell-mediated immune response in mice as a result of the increased numbers of spleen plaque-forming cells, increased titers of sera hemagglutinins, increased numbers of antigen-reactive T-cells, increased number of T-helper cells, and increased production of interleukin 1 (IL-1) by macrophages after pre-treatment with this pure ginsenoside. A later study by Mizuno et al. (1994) showed an increase in Thy1.2 (pan T cell), L3T4 (a marker for helper T cells) and Lyt2 (a marker for cytotoxic T cells) in splenic lymphocytes following the oral administration of a hot water soluble fraction from wild *P. ginseng*. However, the study did not consider which of the ginsenosides was implicated in the cellular immune response. These two studies contradict earlier studies that showed that ginseng saponins had no effect on the immune system (Hikino, 1991), but contradictory results may be attributed to differences in dose levels, composition of extract and/or duration of treatment.

Scaglione et al. (1994) showed in a controlled, single-blind study on alveolar macrophages (AM) from human patients suffering from chronic bronchitis that the daily administration of ginseng extract (G115) increased phagocytic activity and intracellular killing by AM of *C. albicans*. In 1996, Scaglione et al., in a similarly controlled study, showed that G 115 was able to improve the immune response *in*

vivo with 227 human volunteers and could protect against influenza and common cold.

P. ginseng has been used as an anti-ageing tonic drug. Declining responsiveness in lymphocytes is a function of the gradual breakdown in the neuro-endocrino-immuno-system associated with the ageing process (Khansari et al., 1990). Liu et al. (1995) studied the effect of R_{g1} on the lymphocytes of aged people. Their study showed that R_{g1} enhanced the proliferation of lymphocytes stimulated with phytohemagglutinin (PHA): the response of lymphocytes-PHA is used to evaluate cell-mediated immunity, and the degree of lymphocyte proliferation is correlated to ageing. The proliferative response was not as high among young people. The synergistic effect of R_{g1}-PHA was shown to increase the membrane fluidity of the lymphocytes in the elderly as compared to the young people, an effect that has implications in active signal transduction from the cell surface receptors to the inside of the cell.

1.3.2. Polyacetylenes

Hansen and Boll (1986) reported antifungal activity in investigations using polyacetylene-producing plants of the Solanaceae and Apiaceae families. Falcarinone, falcarinol and falcarindiol were shown to be the antifungal compounds. The polyynes of *P. ginseng* demonstrated growth inhibition against Yoshida sarcoma cells (Fujimoto and Satoh, 1987) and cytotoxicity against numerous human tumour cell lines (Matsunago et al., 1994; Matsunaga et al., 1990). *P quinquefolius* polyacetylenes were shown to completely inhibit the growth of leukaemia cells

(Fujimoto et al., 1991). These investigations indicated that the bioactivity of the polyacetylenes was dose-dependent rather than time-dependent, and that the cytotoxicity may be attributed to the decreased fluidity of the cell membrane caused by panaxytriol.

Konoshima (1996) proposed that the significant inhibitory effect on Epstein-Barr virus early antigen (EBV-EA) activation by the crude extract of *Panax notoginseng* (Burk.) FH Chen (Sanchi ginseng), which has more than 10 times the content of R_{g1} than other *Panax* spp. (Tanaka and Kasai, 1984), was a result of the combination of R_{g1} and panaxytriol. Both of the compounds were shown to be inhibitory to EBV-EA activation, but because panaxytriol was cytotoxic at moderate concentrations the inhibitory effect by a smaller amount of the polyacetylene was enhanced by ginsenoside-R_{g1}, which was not cytotoxic.

1.3.3 Polysaccharides

Although there has been little structural elucidation of ginseng polysaccharides, recent studies on *P. ginseng* indicate that they possess primarily immunological properties (Gao et al., 1991), as well as hypoglycaemic activities (Liu and Xiao, 1992; Lien and Gao, 1990; Suzuki and Hikino, 1989a, 1989b; Hikino et al., 1985; Konno et al., 1985). Theses studies have also shown antitumour effects from ginseng polysaccharides, but these are associated with the immunological effects. The biological activities of polysaccharides have been correlated to their molecular weight: the polysaccharide with the greater molecular weight has the greater effect (Gao et al., 1996).

1.4 Rationale and objectives

I believe that traditional knowledge accumulated over millennia by Aboriginal and other peoples shares several features with western science. Experience is recorded in the living library of oral tradition by elders, and knowledge is constantly updated by empirical experience. The present investigation of *P. quinquefolius* was initiated with the view that Aboriginal peoples have used the plant for treatments that suggest it may have significant medicinal activity in carefully controlled studies.

One of the traditional uses of *P. quinquefolius* was as a tonic, a term which has become synonymous in recent literature with the term immunostimulant (Xiao and Liu, 1999). In this study, I investigated the immunostimulating properties of this herb, and the presence of ginsenosides—the presumed bioactive compounds—in wild populations of eastern Canadian Araliaceae.

1.4.1 Objectives of the thesis

The overall objectives of the thesis were the phytochemical characterization of *P. quinquefolius* germplasm resources and an assessment of its immunopharmacological activity. The specific objectives, which are individually addressed in each chapter, were:

1. Validation of HPLC methods. There is a demand in the marketplace for the standardization of phytopharmaceuticals. Our laboratory participated in an international collaborative study to validate an HPLC method that would be used

to quantify ginsenosides, which are used to standardize ginseng and ginseng products.

2. Validation of industrial processing methods. There are two major processing methods for ginseng production: differences in these methods may explain the reported contradictory pharmacological activities. I tested eight different processing methods and compared the varying levels of ginsenoside content obtained through each method.
3. Determine the phytochemical variation in wild *P. quinquefolius* populations. Most ginseng products are now derived from cultivated ginseng, and breeders require phytochemical information on wild populations of American ginseng to improve the cultivated ginseng. I investigated the ginsenoside content in wild populations of eastern Canadian *P. quinquefolius*.
4. Characterize phytochemistry of GP-2 and GP-3 relatives of *P. quinquefolius*. The ethnopharmacological uses of some eastern North American araliaceous relatives of *P. quinquefolius* are similar to the traditional use of ginseng. I investigated the occurrence of ginsenosides in these relatives.
5. Assess the immunostimulating activity of *P. quinquefolius*. The ethnopharmacological use of *P. quinquefolius* suggests immunomodulating activity. I tested its macrophage activating potential, and fractionated it to identify the immunostimulating active principle.

CHAPTER 2

AOAC METHOD VALIDATION FOR GINSENG ANALYSIS

In this chapter, I describe my participation in an international collaborative study to validate an HPLC method for the determination of ginsenosides R_{b1}, R_{b2}, R_c, R_d, R_e and R_{g1} in terms of system suitability, intra-laboratory repeatability, and inter-laboratory reproducibility. This method was used to estimate these ginsenosides in extracts of *Panax ginseng* C.A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (American ginseng) and their commercial products.

Since the Interlaboratory Collaborative Study is to be published in the Journal of the AOAC International shortly, this chapter will be a brief review of my results compared to the study group results. The report of the study is in the Appendix; it provides the background, goals, objectives, materials and methods, results and discussion of the study.

2.1 Results And Discussion

Our laboratory was one of fifteen international laboratories participating in the study. My results are compared to the mean of twelve reporting laboratories in Table 2.1.

For *Panax* species, the repeatability standard deviations ranged from 3.63 – 6.60 % and the reproducibility standard deviations ranged from 6.23 to 12.60 %. For

Table 2.1 Comparison of data from the Interlaboratory Collaborative Study and my determination of ginsenosides in *Panax ginseng*, *Panax quinquefolius*, ginseng product A and ginseng product B.

	Ginsenoside content (mg/kg)[1]					
	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d
P. ginseng						
Average[2]	2810	2925	6478	3663	3433	3211
Study[3]	2940	3230	6970	4700	3550	3060
P. quinquefolius						
Average[2]	1696	11037	32492	3069	504	6477
Study[3]	1720	12300	30100	3220	490	6270
Ginseng A						
Average[2]	214	1853	7130	1778	248	3011
Study[3]	248	2030	6630	1650	240	2830
Ginseng B						
Average[2]	94	234	831	443	435	687
Study[3]	*	*	784	640	403	584

[1] As this study was a quantitative analyses the measurement was in mg/kg.
[2] Results are the average of 3 analyses.
[3] Collaborative results are the average of 12 laboratories.
* There were no analyses of these results since 5 laboratories, including my results, showed discrepancy from the mean of the 12 participating laboratories.

the ginseng products, the repeatability standard deviations ranged from 3.39 – 8.12 % and reproducibility standard derivations ranged from 6.47 – 13.70 %. The average recovery for R_{g1} was 99.9 %, for R_e 96.2 %, and for R_{b1} 92.3%. These results indicate that the method was satisfactory but not robust for the determination of R_{g1} and R_e in the ginseng products at levels below 300 mg/kg. This method was used in subsequent analyses of ginsenoside content for ginseng producers and manufacturers.

CHAPTER 3

PROCESSING METHODS OF GINSENOSIDE ANALYSIS

3.1 Introduction

Ginseng products are marketed as either white or red ginseng. White ginseng is *Panax ginseng* C.A. Meyer (Asian ginseng); however it may also be *Panax quinquefolius* L. (American ginseng) root, which has been peeled, bleached with sulphur dioxide after harvesting, and dried in the sun or artificially dried at 100 – 200 °C. Red ginseng is root that is pre-treated with hot steam before drying and, via the Maillard reaction (a non-enzymatic browning reaction which takes place when components like reducing sugars and amino acids or proteins react together on heating), becomes red (Sonnenborn and Proppert, 1991). These different processing methods result in differences in ginsenosides, and the levels of ginsenosides in the product. For example, only malonyl-ginsenoside-R_{b1}, -R_{b2}, -R_c, and -R_d are present in the white ginseng; in comparison (20*R*)-ginsenoside-R_{g2}, ginsenoside-R_{g3}, (20*S*)- and (20*R*)-ginsenoside-R_{h2}, ginsenoside-R_{s1}, ginsenoside-R_{s2}, quinquenoside-R_1, notoginsenoside-R_1, and panaxatriol are generated during the processing of red ginseng (Shibata et al., 1985).

Identifying the method that provides the highest ginsenoside content would help in both obtaining the highest total ginsenosides and in isolating a specific ginsenoside. Characterizing the products by their commercial processing methods may explain some of the contradictory reports of the actions of ginseng i.e.

hypertensive and hypotensive effects, or stimulatory and depressant activity on the nervous system (Hikino, 1991).

In this chapter, I report the effect of eight different processing methods on ginsenoside content as determined by HPLC analyses. *P. quinquefolius* roots were either freeze dried, dried at 63.5 °C, dried at 95 °C, or steamed for 0.5 – 4 h then dried at 95 °C.

3.2 Materials and Methods

3.2.1 Processing methods

Processed *P. quinquefolius* roots for ginsenoside analyses were provided by Dr. Thomas Francis of the University of Toronto, Toronto, Ontario (Processing methods are A – H in Table 3.1).

3.2.2 Extraction

The roots were milled to no. 40 screen particle size (Thomas-Wiley laboratory mill, Philadelphia, PA). An accurately weighed sample (1 g) of the ground plant root was extracted twice with 20 % aqueous methanolic solution (30 ml) under reflux in a water bath at ~ 55 °C for 30 min each. The combined extract was evaporated (~ 40 °C) to dryness *in vacuo*, and the residue dissolved in 10 ml HPLC-grade methanol. This was then filtered using 0.2 μm nylon filter (Chromatographic Specialties Inc., Brockville, ON) and 5 μl injected into the HPLC system.

3.2.3 Ginsenoside analysis

A Beckman High Pressure Liquid Chromatography (HPLC) system (Beckman Coulter Canada Inc., Mississauga, ON) was used for ginsenoside analysis. The system consists of a module 168 diode-array detector, a module 126 solvent delivery system, a module 502 autosampler, and a computer equipped with System Gold software.

The ginsenosides R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2}, and R_d (from Dr. H Fong, University of Illinois at Chicago and Indofine Chemical Co., Somerville, NJ) were mixed together as pure standards at concentrations of 0.01 - 0.1 mg/ml in HPLC-grade methanol. Analyses based on peak area (AU) were performed and the response factors (mg/ml/AU) calculated and built into the method. Response factors were continuously monitored and updated by bracketing the standard mixture between 6 – 8 samples.

The separation of the ginsenosides was achieved with a reverse-phase Beckman ultrasphere C-18, 5 μm octadecylsilane, 250 x 4.6 mm column (Beckman Coulter Canada Inc., Mississauga, ON) connected to a LiChroCart LiChrospher 100 RP-18 4 x 4 mm, 5 μm guard column (EM Science, Cherry Hill, NJ). The mobile phase was water (A) and acetonitrile (B) at a constant composition of 21 % B from 0 - 20 min, followed by a linear gradient to 42 % B from 20 - 60 min. The column was flushed and equilibrated after each analysis. The flow rate was 1.3 ml/min and the detector was set at 203 nm.

The solvents used in the extraction and the ginsenoside analyses were all HPLC grade: acetonitrile and methanol were Omnisolv (EM Science, Gibbstown,

NJ), and the distilled and de-ionized water was prepared using a Milli-Q Reagent Water System (Millipore Canada Ltd., Nepean, ON).

3.2.4 Statistical analysis

The results were expressed as a mean of % weight per weight (w/w) ± SEM of 5 roots for each of the 8 processing methods. A one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were undertaken: these are considered statistically significant if $P < 0.05$. Statistical significance was determined using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

3.3 Results and Discussion

Figure 3.1 shows the total ginsenoside content resulting from the eight different processing methods. Drying at 95 °C (C), drying at 63.5 °C (B) and pre-steaming for 0.5 h then drying at 95 °C (D) resulted in the highest amounts of total ginsenosides (an average of 5.36 %); these were statistically different from the freeze drying method (A), which resulted in 2.72 % total ginsenosides.

A one-way ANOVA was performed on each of the six ginsenosides to determine the variation of the ginsenoside among the eight processing methods (Table 3.1). Only the changes in concentration of ginsenosides R_{g1}, R_{b1} and R_d were considered significant, and changes in concentration of R_e, R_c and R_{b2} were not statistically significant. The highest amount of R_{g1} (0.20 %) was in the 1 h steam and drying at 95 °C (E) method; the lowest amount (0.04 %) was in the 4 h steam and dry at 95 °C (H). The highest amount of R_e (2.90 %) was in the 3 h steam and dry at

Figure 3.1 Total ginsenoside content in *Panax quinquefolius* L. (American ginseng) roots from 8 processing methods (A-H). Ginsenoside-R_{g1}, -R_e, -R_{b1}, -R_c, -R_{b2}, and -R_d were measured using reverse-phase HPLC. Total ginsenoside content was a mean ± SEM for 5 roots per method. Means followed by the same letter (a - b) are not significantly different in Tukey's multiple comparison test ($P < 0.05$). The processing methods were A-Freeze dry; B-Dry at 63°C; C-Dry at 95°C; D-Steam 0.5 h then Dry at 95°C; E-Steam 1 h then Dry at 95°C; F-Steam 2 h then Dry at 95°C; G-Steam 3 h then Dry at 95°C; and H-Steam 4 h then Dry at 95°C.

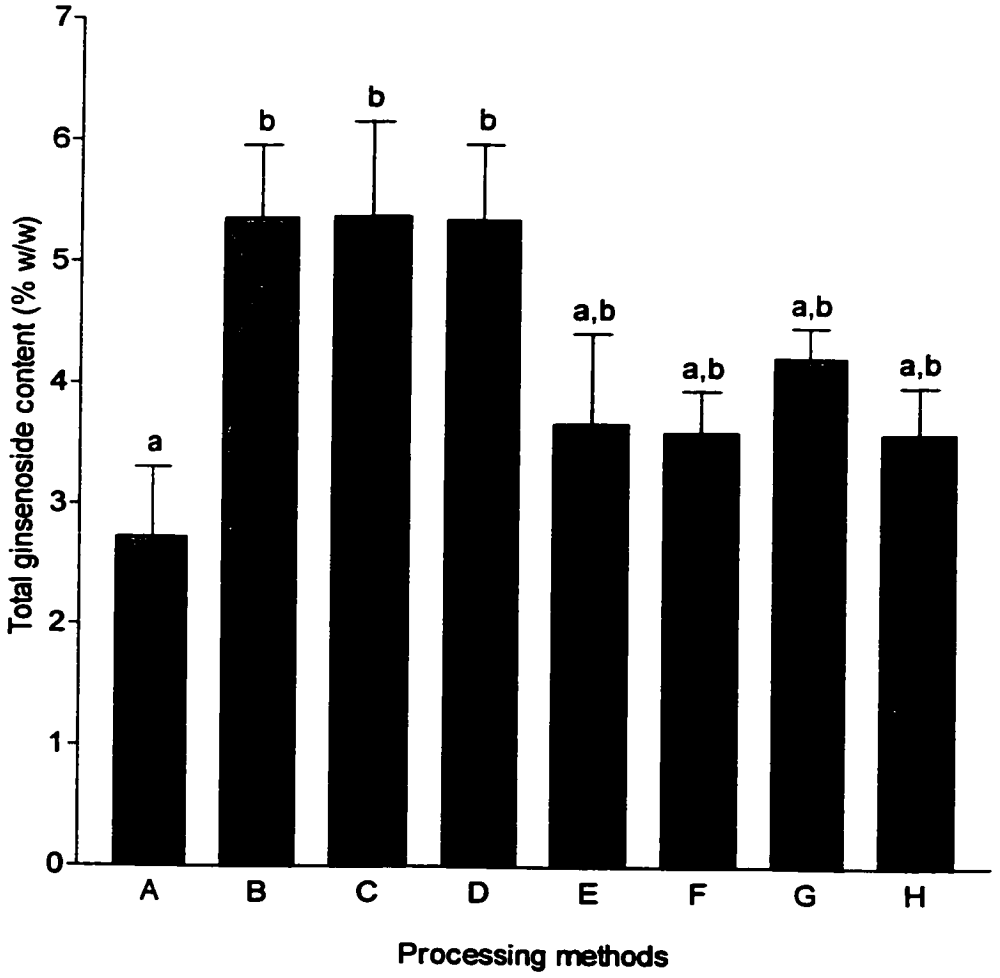

Total ginsenoside content (% w/w)
0
1
2
3
4
5
6
7
a
b
b
b
a,b
a,b
a,b
A
B
C
D
E
F
G
H
Processing methods

Table 3.1 Ginsenoside content in *Panax quinquefolius* L. (American ginseng) roots from different processing methods.

	Processing	Ginsenoside content (% w/w)[1]					
		R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d
A	Freeze Dry	0.11^{ab}	1.22	1.08^{a}	0.11	0.05	0.15^{a}
		(0.01)	(0.21)	(0.32)	(0.02)	(0.01)	(0.04)
B	Dry @ 63.5°C	0.12^{ab}	2.30	2.20^{ab}	0.17	0.06	0.51^{a}
		(0.01)	(0.34)	(0.21)	(0.08)	(0.02)	(0.10)
C	Dry @ 95°C	0.14^{ab}	2.00	2.30^{b}	0.17	0.07	0.64^{a}
		(0.03)	(0.38)	(0.28)	(0.07)	(0.03)	(0.15)
D	0.5 h Steam & Dry @ 95°C	0.12^{ab}	1.90	2.50^{b}	0.17	0.07	0.58^{a}
		(0.02)	(0.21)	(0.23)	(0.05)	(0.02)	(0.22)
E	1 h Steam & Dry @ 95°C	0.20^{a}	0.92	1.90^{ab}	0.16	0.05	0.48^{a}
		(0.07)	(0.28)	(0.48)	(0.04)	(0.01)	(0.12)
F	2 h Steam & Dry @ 95°C	0.13^{ab}	1.50	1.10^{a}	0.08	0.05	0.70^{a}
		(0.02)	(0.16)	(0.07)	(0.03)	(0.02)	(0.12)
G	3 h Steam & Dry @ 95°C	0.06^{ab}	2.90	1.60^{ab}	0.06	0.05	0.38^{a}
		(0.01)	(0.44)	(0.16)	(0.04)	(0.02)	(0.07)
H	4 h Steam & Dry @ 95°C	0.04^{b}	1.90	1.60^{ab}	0.09	0.04	0.28^{a}
		(0.01)	(0.34)	(0.08)	(0.02)	(0.01)	(0.03)

[1] Percentages based on weighed ground root material.
Results were means from 5 roots for each method measured by reverse-phase HPLC. The values in brackets below the mean are ± SEM. For each column, different letters following the mean indicate differences determined by Tukey's multiple comparison tests ($P < 0.05$).

95 °C (G); the lowest amount (0.92 %) was in the 1 h steam and dry at 95 °C (E). The highest amount of R_{b1} (2.50 %) was in the 0.5 h steam and dry at 95 °C (D); the lowest amount (1.08 %) was in the freeze drying (A) method, which was statistically similar to the ginsenoside level in the 2 h pre-steam followed by the heat treatment (F). The highest amount of R_c (0.17 %) was in the drying at 63.5 °C (B), which was the same amount for drying at 95 °C (D) and 0.5 h steam and dry at 95 °C (E); the lowest amount (0.06 %) was in the 3 h steam and dry at 95 °C (G). The highest amount of R_{b2} (0.07 %) was in the drying at 95 °C (C), which was the same as in 0.5 h steam and dry at 95 °C (D); whereas the lowest amount (0.04 %) was in the 4 h steam and dry at 95 °C (H). The highest amount of R_d (0.70 %) was in the 2 h steam and dry at 95 °C (F); the lowest amount (0.15 %) was in the freeze-drying (A) method.

The overall trend shown in Table 3.1 is that drying at high temperature, at 95 °C rather than at 63 °C, ensures the best quantities of R_{g1}, R_{b1}, and R_d, and that the pre-steaming for 0.5 h then drying at 95 °C (D) method provides statistically similar amounts of the ginsenosides as the high heat treatments. Drying at 95 °C (C) preserves higher amounts of R_{g1} and R_d compared to the other high-level processing methods. Freeze drying (A) yielded the lowest total amount of ginsenosides and overall yielded the lowest amount of R_{b1} and R_d. The high amount of total ginsenosides found in 3 h steam and dry at 95 °C (G) was attributed to a high amount of R_e; this was likely an error in quantification since steaming for less times appeared to produce lower amounts of this compound.

The pre-steam treatment of longer than one half hour yields smaller amounts of total ginsenosides as compared to drying at high temperature. This suggests that the alleged improved pharmacological activity of red ginseng (Choi et al., 1988) may be the result of lower amounts of R_{g1}, R_{b1} and R_d. A result of increasing the steaming period was an increase in the ratio of R_{b1} to R_{g1}: the ratios increase from 9.5 at 1 h pre-steaming to 40 at 4 h pre-steaming. The individual ginsenosides R_{b1} and R_{g1} have opposing effects: R_{b1} has been shown to be a central nervous system depressant, and to have antipsychotic, hypotensive, antistress, anti-inflammatory, gastro-intestinal motility activating, and antihaemolytic actions; in contrast R_{g1} has been shown to stimulate the CNS, and have hypertensive, antifatigue and stress ulcer aggravating actions (Hikino, 1991). Alternatively, the compounds (20*R*)-ginsenoside-R_{g2}, ginsenoside-R_{g3}, (20*S*)- and (20*R*)-ginsenoside-R_{h2}, ginsenoside-R_{s1}, ginsenoside-R_{s2}, quinquenoside-R_1, notoginsenoside-R_1 and panaxatriol that are produced from the pre-steaming process (Shibata et al., 1985) may be red ginseng's bioactive components.

The amount of R_{b1} typically determines the total amount of the ginsenosides in the root. Our resulting range of 40 - 47 % of the total amount of ginsenosides for R_{b1} in these processing methods is consistent with those in the literature (Smith et al., 1996; Soldati and Sticher, 1980).

This study showed that the quantity of ginsenosides is affected by the processing methods. To obtain the largest quantities of total ginsenosides, and specifically R_{b1}, R_{g1} and R_d, drying the root at 95 °C is the most promising method.

CHAPTER 4

PHYTOCHEMISTRY OF *PANAX QUINQUEFOLIUS* L. (AMERICAN GINSENG)

4.1 Introduction

Variation in ginsenoside concentrations has been characterized for the different wild populations of the Asian *Panax* spp. (Tanaka and Kasai, 1984; Zhou et al., 1975), and cultivated *Panax quinquefolius* L. (American ginseng) (Smith et al., 1996). Fushimi et al. (1996) was the first to use 18S ribosomal RNA gene sequences to show that there are different base substitutions at 4 nucleotide positions in the roots of *P. quinquefolius* and *P. ginseng*. And research on genetic diversity in *P. quinquefolius* using randomly amplified polymorphic DNA (RAPD) marker analysis also showed significant genetic diversity in cultivated ginseng roots (Bai et al., 1997).

P. quinquefolius' natural range is throughout the eastern temperate forest region of North America, from southern Quebec to Minnesota, and south from Oklahoma to Georgia (Argus et al., 1984). The populations occur in mature deciduous forests dominated by *Acer saccharum* Marsh. (Sugar maple) and other hardwoods. The halictid bees, *Dialictus* sp. and *Evylaeus* sp., and the syrphid hover-flies, *Toxomerus marginatus* Say, *Mesograpta boscii* Macq., and *Melanostoma mellinum* L. are the principal pollinators of this species. However, they are not necessary for fruit production, since flowers within the same inflorescence may cross-fertilize by wind pollination (Catling and Spicer, 1995; Schlessman, 1985;

Lewis and Zenger, 1983). Bee and wind pollinators are not sufficient to bridge the distance between extant populations: insect pollination occurs within 300 meters of the parent plant; whereas pollen spread by the wind falls generally within 50 meters of the parent plant (Raven et al., 1986). Thus, the fragmentation of the mature hardwood forests by human activity, the over-harvest of the roots, and the autogamous nature of the organism have contributed to its patchy distribution and the poor gene flow between populations of *P. quinquefolius*.

Roots from the northern populations of *P. quinquefolius* are considered of higher quality and sold for a higher price than those from the southern populations of *P. quinquefolius* (Carlson, 1986). Since ginsenosides are considered the main bioactive compounds in the genus (Shibata et al., 1985), this suggests that the roots from northern populations of *P. quinquefolius* may have different ginsenoside content from the southern populations. However, little information is available on variation in the wild populations. Establishing the nature of phytochemical variation would be of interest for several reasons. Information on ginsenoside levels in wild ginseng is important for quality assurance programs such as the American Botanical Council's Ginseng Evaluation Project (Hall et al., 2001; Fitzloff et al., 1999) so that claims of pure root products in commercial material can be compared to authentic ginseng. Establishing the variation in wild populations could provide information to growers about the best seed sources. Establishment of superior genotypes would provide an economic incentive for the rehabilitation of forest ginseng, and cultivation of American ginseng genotypes with the appropriate ginsenoside levels in agricultural lands would conserve wild *P. quinquefolius* populations.

The objective of this study was to determine the phytochemical variation in wild *P. quinquefolius* populations. Because ginseng occurs in widely separated and highly patchy forest habitats, it was hypothesized that genetic isolation of populations may be reflected in distinct chemotypes of *P. quinquefolius*.

4.2 Materials and Methods

4.2.1 Plant material

Plants were collected in the autumn of 1997 – 1999 from 6 sites in Ontario and 1 site in Quebec. Other roots were donated from sites in Ontario, Maine, Vermont and Wisconsin. Voucher specimens of *P. quinquefolius* (Table 4.1) have been deposited in the herbarium at the University of Ottawa. Because *P. quinquefolius* is considered endangered, all collections were made in collaboration with conservation authorities in such a way as to not harm the existing populations. The conservation authorities allowed only limited collections.

4.2.2 Extraction and analyses of ginsenosides

4.2.2.1 Extraction

The plants were dried on the laboratory bench at 20 - 22 °C for 4 - 6 weeks. The different plant parts (leaves, stem, and root/rhizome) were then milled to no. 40 mesh particle size (Thomas-Wiley laboratory mill, Philadelphia, PA). An accurate weighed sample (1 g) of the ground plant material was extracted twice with 20 % aqueous methanolic solution (30 ml) under reflux in a water bath at ~ 55 °C for 30 min each. The combined extract was evaporated (~ 40 °C) to dryness *in vacuo*, and

Table 4.1 Collection data for *Panax quinquefolius* L. (American ginseng) populations.

Voucher No.	Population	Site (County/State)[1]	Number of individuals[2]
19501	1	Wisconsin	2 (donated)
19502	2	Lanark	3
19503	3	central Frontenac	5
19504	4	Vermont	2 (donated)
19505	5	Maine	3 (donated)
19506	6	Haldiman-Norfolk	6 (donated)
19507	7	North Frontenac-1	5
19508	8	North Frontenac-2	3
19509	9	Iberville	5
19510	10	Lennox and Addington	4 (donated)

[1] Precise location by GPS is not provided to protect the wild populations.

Donations were pieces of a root or the main root without the rhizome or the rootlets.

the residue dissolved in 10 ml HPLC-grade methanol. This was filtered using a 0.2 μm nylon filter (Chromatographic Specialties Inc., Brockville, ON) and 5 μl injected into the HPLC system.

4.2.2.2 Ginsenoside analysis

A Beckman High Pressure Liquid Chromatography (HPLC) system (Beckman Coulter Canada Inc., Mississauga, ON) was used for ginsenoside analyses. The system consists of a module 168 diode-array detector, a module 126 solvent delivery system, a module 502 autosampler, and a computer equipped with System Gold software.

The ginsenosides R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2}, and R_d (from Dr. H Fong, University of Illinois at Chicago and Indofine Chemical Co., Somerville, NJ) were weighed accurately and mixed together as pure standards at concentrations of 0.01 - 0.1 mg/ml in methanol. Analyses based on peak area (AU) were performed and the response factors (mg/ml/AU) calculated and built into the method. Response factors were continuously monitored and updated by bracketing the standard mixture between 6 – 10 samples.

The separation of the six major ginsenosides found in *P. quinquefolius*, that is R_{g1}, R_e, R_{b1}, R_c, R_{b2}, and R_d, was achieved with reverse-phase Beckman ultrasphere C-18, 5 μm octadecylsilane, 250 x 4.6 mm column (Beckman Coulter Canada Inc., Mississauga, ON) connected to a LiChroCart LiChrospher 100 RP-18 4 x 4 mm, 5 μm guard column (EM Science, Cherry Hill, NJ). The mobile phase was water (A) and acetonitrile (B) at a constant composition of 21 % B from 0 - 20 min followed by

a linear gradient to 42 % B from 20 - 60 min. The column was flushed and equilibrated after each analysis. The flow rate was 1.3 ml/min and the detector was set at 203 nm.

All the solvents were HPLC-grade: acetonitrile and methanol were Omnisolv products (EM Science, Gibbstown, NJ) and the distilled and de-ionized water was prepared using a Milli-Q Reagent Water System (Millipore Canada Ltd., Nepean, ON).

4.2.3 Canonical analyses

To determine the significance of phytochemical variation within and among ten wild populations of *P. quinquefolius*, canonical analyses (CA) were performed using the ginsenoside content of the roots. The ginsenosides R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2}, and R_d were quantitative characters or variables, and the populations were the classification criteria. All statistical analyses were performed using SAS procedures (SAS Institute Inc., 1997).

4.2.4 Statistical analyses

One-way or two-way analyses of variance (ANOVA) of the data were carried out using General Linear models procedures of SYSTAT 9 for Windows (SPSS Science Inc., 1998). Tukey's multiple comparison tests provided further tests when significant *P* statistics resulted from an initial ANOVA. All results were expressed as mean of % w/w ± SEM and considered statistically significant if $P < 0.05$.

4.3 Results

4.3.1 Plant collection

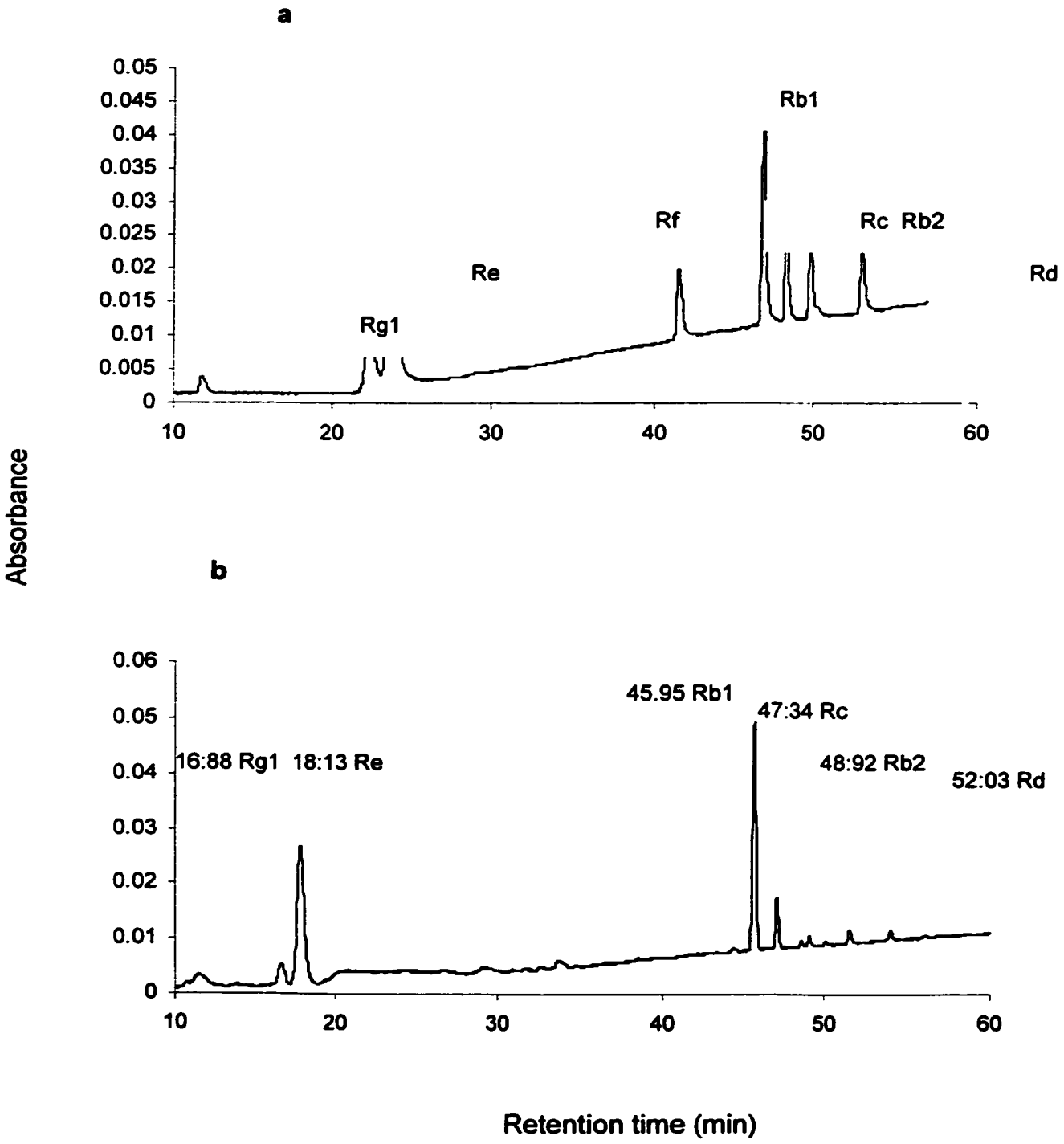
P. quinquefolius roots from ten locations were obtained for the study. As listed in Table 4.1, populations 1, 4, and 5 are roots from American locations; population 9 is from a Quebec location; the rest of the populations are from Ontario locations. All the roots from American locations, as well as roots from populations 3, 6 and 10 were donations. The roots from USA donors were already ground; the roots donated by Canadian sources generally had no root hairs and no rhizome. The donors provided ages of the roots and their population locations.

The plants collected were assessed on the basis of age. Since stem height, the number of leaflets and the number of leaves increase with age (Anderson et al., 1993), these were used as predictors of the age of the *P. quinquefolius* plants. Counting the stem scars on the rhizome (Carpenter and Cottam, 1982) verified the age of the plant. Plants were also collected in the fall, so that only plants with many berries were selected and this selection afforded the opportunity to plant the berries on or near the collection site.

4.3.2 Ginsenoside analysis

Reverse-phase HPLC analyses were completed on all the plant parts for 19 whole plants and an additional 19 roots. Figure 4.1a shows a chromatogram of the

Figure 4.1 Chromatograms from HPLC analysis showing the peaks and the retention times of (a) the ginsenoside standard mix and (b) a typical sample of a *Panax quinquefolius* L. (American ginseng) root.


a
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Rb1
Rf
Rc Rb2
Re
Rd
Rg1
10
20
30
40
50
60
Absorbance
b
0.06
0.05
0.04
0.03
0.02
0.01
0
45.95 Rb1
47:34 Rc
16:88 Rg1
18:13 Re
48:92 Rb2
52:03 Rd
10
20
30
40
50
60
Retention time (min)

standard mix containing ginsenoside-R_{g1}, -R_e, -R_f, -R_{b1}, -R_c, -R_{b2}, and –R_d showing typical separation. Figure 4.1b shows a chromatogram of a four-year old *P. quinquefolius* root identifying the ginsenosides and their retention times. Baseline separation of all ginsenosides was achieved, and the high R_{b1}/R_{g1} ratio and the absence of R_f confirm that the plants were authentic *P. quinquefolius*.

Figure 4.2 shows the range in total ginsenoside content among 38 roots. Sixty percent of the roots had total ginsenoside content between 3.0 – 6.9 %. No root had less than 1 %, and 13 % had surprisingly high total ginsenoside content (> 10 %).

Table 4.2 shows the results of the reverse-phase HPLC ginsenoside analyses of the different plant parts of 19 plants and additional 19 roots. A one-way ANOVA with Tukey's posttest was performed on each plant part. Leaves had similar content of R_e, R_{b2} and R_d, and these were significantly higher than the other ginsenosides. The mean and SEM of ginsenoside content observed in these leaves was 0.14 ± 0.03 % for R_{g1} to 1.08 ± 0.19 % for R_d. There were no significant differences in the individual ginsenoside content of stem; the content range in the stem was from 0.05 ± 0.04 % for Rc to 0.53 ± 0.10 % for R_e. In contrast, there was more variation in ginsenoside content in the roots: R_{b1} was significantly higher than any other ginsenoside. R_e was present in half the amount of R_{b1}, and was statistically similar to R_{g1}, but was statistically more abundant than R_c, R_{b2} and R_d. R_{b2} was present in statistically the lowest amount of all the ginsenosides. The content (mean and SEM) in the root was 0.09 ± 0.02 % for R_{b2} and 2.81 ± 0.36 % for R_{b1}. Because of the low

Figure 4.2 Range of total ginsenoside content among 38 *Panax quinquefolius* roots.

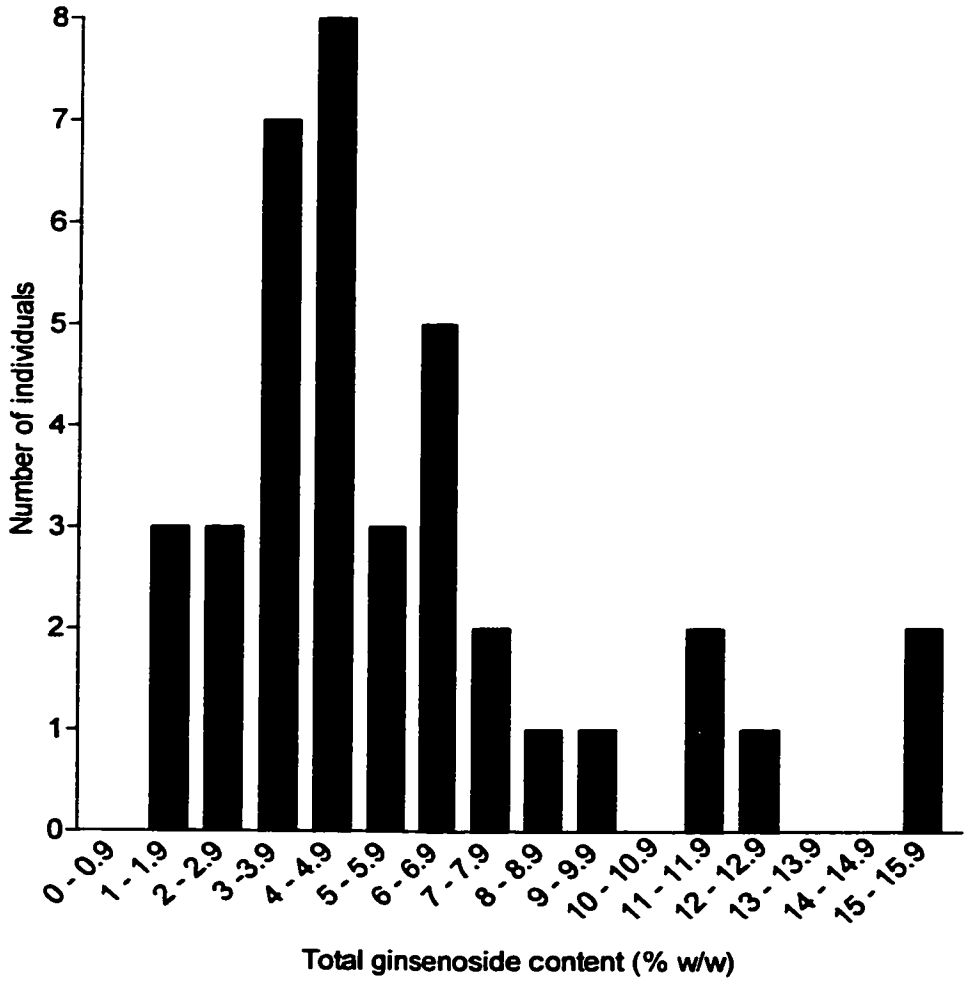

Number of individuals
0
1
2
3
4
5
6
7
8
0 - 0.9
1 - 1.9
2 - 2.9
3 - 3.9
4 - 4.9
5 - 5.9
6 - 6.9
7 - 7.9
8 - 8.9
9 - 9.9
10 - 10.9
11 - 11.9
12 - 12.9
13 - 13.9
14 - 14.9
15 - 15.9
Total ginsenoside content (% w/w)

Table 4.2 Distribution of ginsenoside-R_{g1}, -R_e, -R_{b1}, -R_c, -R_{b2}, and –R_d in dried *Panax quinquefolius* leaves, stem and root as measured using reverse-phase HPLC. Ginsenoside content is a mean ± SEM of 19 leaves, 19 stems and 38 roots. Means in each row followed by the same letter (a – d) are not significantly different in Tukey's multiple comparison tests. Similarly, the same letter (x or y) in the total ginsenosides content column denotes no statistical significant difference between the plant parts.

	Content (% w/w)[1]						
	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d	Total
Leaves	0.14 ± 0.03 ^{b}	0.93 ± 0.14 ^{a}	0.17 ± 0.05 ^{b}	0.18 ± 0.03 ^{b}	1.04 ± 0.15 ^{a}	1.08 ± 0.19 ^{a}	3.33 ± 0.48 ^{x}
Stem	0.12 ± 0.05 ^{a}	0.52 ± 0.10 ^{a}	0.11 ± 0.04 ^{a}	0.05 ± 0.04 ^{a}	0.36 ± 0.17 ^{a}	0.46 ± 0.22 ^{a}	1.62 ± 0.55 ^{x}
Root	0.94 ± 0.24 ^{b,c}	1.42 ± 0.17 ^{b}	2.81 ± 0.36 ^{a}	0.42 ± 0.06 ^{c,d}	0.09 ± 0.02 ^{d}	0.29 ± 0.08 ^{c,d}	5.78 ± 0.59 ^{y}

[1] Percentages were calculated based on the weighed ground plant material.

number of plant samples (19) a frequency distribution of ginsenoside content was not determined. However, the lowest and highest amount of total ginsenosides were 0.03 – 10.15 % for stem, 0.18 – 6.57 % for leaves, and 1.14 – 15.95 % for roots.

Roots had statistically more total ginsenosides than the leaves ($P < 0.05$), and the stems ($P < 0.001$). In a separate ANOVA (not shown), the higher ginsenoside content in the roots is attributed to significantly higher content of R_{b1}, R_c and R_{g1}.

4.3.3 Phytochemical variation among populations

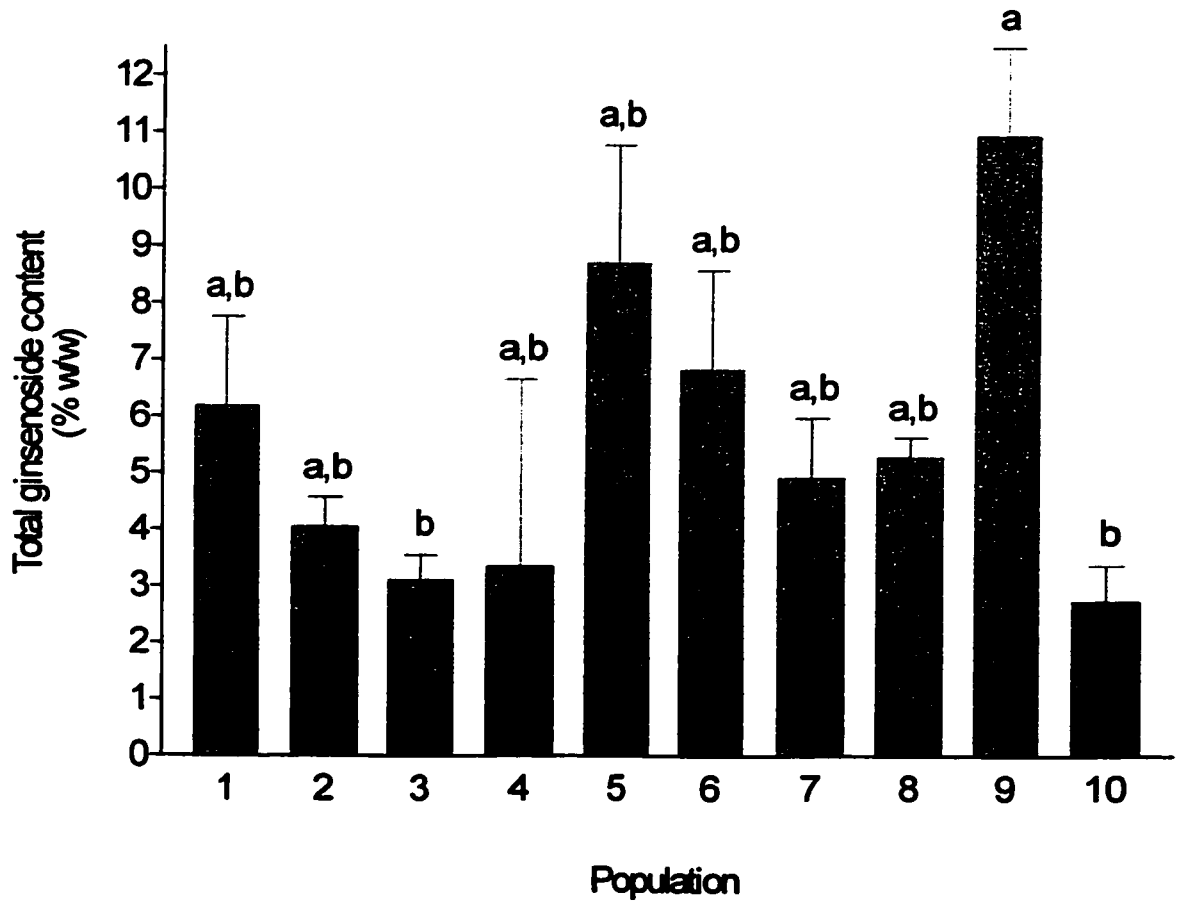
4.3.3.1 Population results by ANOVA

As there was considerable variation in the ages of the roots within a population (for example, a population could have roots aged from 4 – 37 years of age), the roots were categorized into three age groups: 5 or less, 6 – 10, and 11+. In all three groups, the ginsenosides evaluated were R_{g1}, R_e, R_{b1}, R_c, R_{b2} and R_d. The range was between R_{b1}, which had the highest content at 2.81 ± 0.36 %, and R_{b2}, which had the lowest content at 0.09 ± 0.01 %.

There was significant variation (one-way ANOVA ($P < 0.004$)) in ginsenoside content by population (Figure 4.3). There are two populations that are significantly different ($P < 0.01$) from each other: populations 3 and 9, and populations 9 and 10. The means of the total ginsenoside content ranged from 2.73 ± 0.63 % for population 10 and 3.11 ± 0.44 % for population 3 to 10.93 ± 1.56 % for population 9.

When age class was considered in the analysis (not shown), there was no significant effect from that shown with the ANOVA using just the ginsenosides and the populations.

Figure 4.3 Total ginsenoside content in the roots of 10 wild populations of *Panax quinquefolius*. Ginsenoside-R_{g1}, -R_e, -R_{b1}, -R_c, -R_{b2}, and –R_d were measured using reverse-phase HPLC. Total ginsenoside content is a mean ± SEM for 2 - 5 roots per population. Means followed by a same letter (a – b) are not significantly different in Tukey's multiple comparison test ($P < 0.05$) between the populations.


Total ginsenoside content (% w/w)
12
11
10
9
8
7
6
5
4
3
2
1
0
a,b
a,b
b
a,b
a,b
a,b
a,b
a
b
1
2
3
4
5
6
7
8
9
10
Population

4.3.3.2 Canonical analysis

The previous ANOVA and Tukey's analyses were based on individual ginsenosides to determine significant differences between organs and populations. Canonical analysis (CA), however, is appropriate as a multivariate approach used to evaluate the overall effect of all ginsenosides as discriminators of unique populations. Mean differences between populations (squared Mahalanobis distances) from the CA are presented in Table 4.3. The distances ranged from 0.915 between populations 2 and 7 to 15.725 between populations 2 and 9. Tests of significance for the pair-wise distances between populations indicate that populations 3 and 9, populations 6 and 9, and populations 10 and 9 were significantly different. These results are similar to the previous multiple range data generated with the total ginsenosides (Figure 4.3).

When age was added as a variable in the foregoing CA (not shown), only one other phytochemically different population pair was observed: populations 4 and 9.

The calculated eigenvalues and the proportion of total variance for the 7 ginsenosides are presented in Table 4.4. These first four interactions accounted for 93 % of the total variability. The differences between the populations are characterized by the within-canonical structure presented in the Table 4.5. CAN1 explained 52 % of the total variance, and was mainly correlated to R_c (0.798) and R_{b1} (0.682). CAN2 accounted for 23 % of the total variance, and was mainly correlated to Rd (0.585) and R_{b1} (0.582). CAN3 explained 12 % of the total variance, and was mainly correlated to R_{b2} (0.438) and R_{g1} (0.344).

Table 4.3 Mahalanobis distances (above the diagonal) and test of significance (*P* value) (below the diagonal) from the canonical analysis of ginsenoside content in roots of 9 wild populations of *Panax quinquefolius*.

	Populations								
	2	3	4	5	6	7	8	9	10
2	-	2.589	2.838	5.469	4.078	0.915	2.626	10.971	1.561
3	0.829	-	4.603	8.684	7.067	2.777	4.329	**11.891**	3.849
4	0.905	0.686	-	6.591	8.918	4.026	2.839	12.896	2.893
5	0.517	0.175	0.539	-	9.197	4.228	4.267	4.014	6.384
6	0.520	0.123	0.226	0.097	-	4.628	6.964	**14.443**	6.272
7	0.984	0.683	0.719	0.538	0.256	-	1.803	7.159	2.859
8	0.867	0.578	0.905	0.663	0.206	0.906	-	7.313	4.135
9	0.067	**0.026**	0.097	0.570	**0.004**	0.105	0.214	-	**15.725**
10	0.934	0.491	0.853	0.285	0.120	0.601	0.552	**0.004**	-

Table 4.4 Eigenvalues and percentage of variation explained by the axes.

Variable	Eigenvalue	% Variance
R_{g1}	1.955	52
R_e	0.892	24
R_f	0.441	12
R_{b1}	0.206	5
R_c	0.149	4
R_{b2}	0.077	2
R_d	0.027	1

Table 4.5 Within-canonical structures for the 7 ginsenosides in 9 populations of *Panax quinquefolius* on four canonical axes.

Ginsenoside	CAN1	CAN2	CAN3	CAN4
R_{g1}	0.162	0.270	0.344	0.386
R_e	0.142	0.455	-0.122	-0.518
R_f	0.037	-0.125	-0.641	0.393
R_{b1}	0.682	0.582	-0.019	0.026
R_c	0.798	0.010	0.250	-0.085
R_{b2}	0.215	-0.048	0.438	0.628
R_d	-0.059	0.585	0.112	-0.176

4.4 Discussion

This is the first report of naturally occurring ginsenoside levels in a significant survey of North American ginseng populations. When roots of similar age are compared, the total ginsenoside content is similar to levels reported for cultivated *P. quinquefolius* (Table 4.6). The wild populations do, however, include roots of exceptional age and ginsenoside content. This may have led to the misconception that "woods grown" or wild ginseng is medicinally more potent. This does not appear to be supported by our data; an important conclusion is that cultivated ginseng has levels of medicinally important ginsenosides comparable to those of wild ginseng.

Foster (1996) noted that wild *P. quinquefolius* root has higher levels of ginsenosides than the cultivated root. However, Tanaka (1987) reported no significant difference in saponin composition between wild and cultivated *P. ginseng*. These discrepancies may result from variation in collection times and in the tissues used for the ginsenoside quantifications. Kim et al. (1981) reported seasonal variation in saponin and carbohydrate content in *P. ginseng* roots: whereas saponin content peaked in the summer, carbohydrate content, which was mainly due to the increase of sucrose, peaked in winter. Furthermore, if lateral roots and root hairs are removed from the main root, as they are in most commercial products, then the ginsenoside content is different. Soldati and Sticher (1980) reported that in *P. ginseng* root hairs has twice as much ginsenoside content as lateral roots, which, in turn, have three times as much ginsenoside content as the main root. These authors also reported variation in the kind of ginsenoside present: R_e was the prominent

Table 4.6 Comparison of ginsenoside content in cultivated and wild *Panax quinquefolius*.

	Content (% w/w)[1]						
	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d	Total
Leaves							
Li et al., 1996[2]	0.28	1.56	0.23	0.32	0.62	1.74	4.18
This study	0.14	0.93	0.17	0.18	1.04	1.08	3.33
Root							
Court et al., 1996[3]	0.16	1.53	1.93	0.25	0.04	0.41	4.32
Li et al., 1996[4]	0.18	1.10	1.22	0.18	0.02	0.29	3.00
Cultivated root[5]	0.25	1.75	1.88	0.36	0.13	0.48	4.85
This study[6]	0.94	1.42	2.81	0.42	0.09	0.29	5.78

[1] Percentages were calculated based on the weighed ground plant material.
[2] Average from leaves of 54 *P. quinquefolius* plants collected from commercial fields in British Columbia in early September 1994.
[3] Average from roots of 54 4-year old *P. quinquefolius* plants collected from field experiments at the Pest Management Research Centre, Delhi, ON in early September 1994 and 1995.
[4] Average from roots of 8 4-year old *P. quinquefolius* plants collected from commercial fields in British Columbia in September 1994
[5] Average of 12 4-year old cultivated *P. quinquefolius* roots collected in late September 1998 from Northern Lights Ginseng Farm, Quyon, QC.
[6] Refer to Table 4.2.

ginsenoside in root hairs, whereas R_{b1} was prominent in lateral roots and R_{g1} was the prominent ginsenoside in the main root. We have also observed these differences in our investigations with cultivated *P. quinquefolius* (not shown).

The difference in ginsenoside content between different plant parts of a plant shown in Table 4.2, was also reported by others: the decreasing order of ginsenoside content was bud-flower > leaf > root > rhizome > seed (Nah, 1997; Liu and Xiao, 1992). Li et al. (1996) reported that *P. quinquefolius* leaves contain mainly Re and Rd, whereas the roots contain mainly R_{b1} and R_e. They suggested that the leaves mature and accumulate these compounds, as there is a corresponding decrease in the roots. Although our root results are similar to those in the literature (Li et al., 1996; Shibata et al., 1985), our leaves results add R_{b2} as a significant saponin (Li et al., 1996) (Table 4.7). In contrast, although the major ginsenosides have been identified in the stem (Chen et al., 1981), their quantification and variation is reported here first.

The age of the plant also affects ginsenoside concentrations. Court et al. (1996) showed that ginsenoside concentrations increase with root age: changes were specifically attributable to R_{b1}, mR_{b1}, R_e and R_o. They recommended a harvest of the ginseng crop at age four rather than three years to obtain a higher amount of ginsenosides. Soldati and Tanaka (1984) showed similar results for cultivated *P. ginseng*; moreover, their study determined that there is no marked increase in ginsenoside concentration after the fifth summer. Our results from the ANOVA and the CA, using age as a variable, did not demonstrate a markedly different result from the analyses without the age variable. This may have been a result of the limitations

of our study in that we had insufficient samples. Our samples also had considerable age variation in some populations (populations 2, 7, and 8) and no variation in others (populations 1, 4 and 6).

It was hypothesized that we would observe significant phytochemical variation between populations. This was supported by the ANOVA of total ginsenosides (Figure 4.3) and the CA of seven ginsenosides (Table 4.3) that showed several examples of statistically different levels between certain populations. Population 9, which represented a population in Iberville county in Quebec, was established as the most different from the other populations. Populations 3, 7 and 8, which were all from Frontenac county in Ontario, were shown to be distinct populations.

One limitation of the study is the low sample size per population, which was a result of the endangered status of *P. quinquefolius* and the collection limits set by conservation authorities. We believe that if more samples could be taken, SEM would have been smaller and more powerful statistical differences identified.

An important feature of the study was to provide baseline data on authentic *P. quinquefolius*. One of the challenges of the Ginseng Evaluation Project's (GEP) evaluation of 414 commercial products (Hall et al., 2001; Fitzloff et al., 1999) was the lack of information on authentic material. The results in Figure 4.2 show the ranges of ginsenoside in wild crafted material. For example, no material was observed to have less than 1 % total ginsenosides. The large number (20 %) of commercial products tested in the GEP claiming ginseng root content and with less than 1 % ginsenosides suggests adulteration in commercial products. Ginseng producers can compare their product with authentic levels described in Figure 4.2.

The variation observed in ginsenoside concentration in the different accessions may be of both environmental and genetic origin (Baldwin and Preston, 1999; Bazzaz et al., 1987). Influences from the environment, such as soil nutrients and texture, humidity of soil and air, and light conditions, affect the chemical profile of populations. As a result, there is variation in the concentration of ginsenosides found in plants of the same species. For example, R_{b1}, R_c, and R_d have been shown to be most susceptible to varying environmental conditions (Li et al., 1996). The influence of soil, nutrient and water is best evaluated in highly replicated and uniform conditions of cultivation. Therefore, in this study we did not attempt to measure soil or other environmental parameters, since it was unlikely that firm conclusions could be drawn on the basis of such a small sample size. Genetic variation also plays a role in ginsenoside concentration. Dr. A. Nault, of the Montreal Biodome, and Dr. D. Gagnon, of the Université du Quebec à Montréal have studied genetic variation in wild populations of *P. quinquefolius,* including some of the populations studied here. Preliminary data suggest genetic differences between populations that may reflect a history of fragmentation (isolation) and size reduction through past harvesting (D. Gagnon, personal communication).

CHAPTER 5

PHYTOCHEMISTRY OF EASTERN CANADIAN ARALIACEAE

5.1 Introduction

There are fifty-six genera and twelve hundred and ninety species of Araliaceae worldwide. Although most members of this predominantly woody plant family occur in tropical America and Indo-Malaya, there are three temperate genera: *Aralia*, *Oplopanax* and *Panax* (Wielgorskaya, 1995).

Araliaceous plants have traditionally been used as medicines. *Panax ginseng* C.A. Meyer (Asian ginseng), the best-known member of this family, has a long history of medicinal use by East Asians, and is used in both East and West to promote good health by stimulating the metabolism, and as a tonic during convalescence (Li, 1995; Raman and Houghton, 1995; Fulder, 1980). In allopathic medicine several medicinal and therapeutic effects of ginseng have been established through *in vitro* studies, animal trials and clinical studies. These include such immunostimulating studies as the augmentation of murine lymphocyte proliferation (Mizuno et al., 1994) and natural killer (NK) cell function (Jie et al., 1984), and, in human studies, the increase of neutrophil function, CD4 cell count and NK cell function (See et al., 1997; Scaglione et al., 1996; Scaglione et al., 1990).

Panax quinquefolius L. (American ginseng), a close relative of *P. ginseng*, was once prolific in the understory of eastern North American hardwood forests (Charron and Gagnon, 1991; Lewis and Zenger, 1982), but due to over-harvesting and habitat destruction is now an endangered species both in Canada and through

much of its natural range in the United States of America (Nault and White, 1999; Robbins, 1998; White, 1988).

Traditionally, the Iroquois used *P. quinquefolius* root infusions to treat fever; the Maritime Indians used the root as a blood detergent; and the Ojibwa used the root to alleviate stomach pain (Arnason et al., 1981). Other Aboriginal peoples of North America used the root to relieve childbirth pain, cure nosebleed, treat shortness of breath, strengthen mental powers, increase the fertility of women, and as a general tonic (Borchers et al., 2000; Foster, 1996) (Table 5.1).

Ginsenosides are the major chemical constituents of *P. quinquefolius* (Tanaka, 1990; Shibata et al., 1985). Other dammarane-type triterpene oligo-glycosides are quinquenosides I, II, III, IV, and V (Yoshikawa et al., 1998). Other phytochemicals include the flavonoids kaempferol-3,7-dirhamnoside and kaempferol-3-gluco-7-rhamnoside (Lee and Der Marderosian, 1988a, 1988b), the sesquiterpenes α-panasinene, β-panasinsene and β-neoclovene (Iwabuchi et al., 1987), the polyacetylenes panaxynol, panaxydol, panaxytriol, heptadeca-1,8-diene-4,6-diyne-3,10-diol, PQ-1, PQ-2, PQ-3 (Fujimoto et al., 1991) and 6'-*O*-acetyl-ginsenoside-R_{g1} (Yoshikawa et al., 1998), and the polysaccharides quinquefolans A, B, and C (Oshima et al., 1987).

Panax trifolius Linn. (Dwarf ginseng), a spring ephemeral, is found in the understory of eastern North America hardwood forests (Gleason, 1968). Seneca women used the root to alleviate childbirth pains (Isaacs, 1977), and other Amerindians also used the root to treat headaches, shortness of breath, fainting and nervous debility; they

Table 5.1 Uses of some Araliaceae by species and usage category.

Usage category[1]	*Aralia nudicaulis*[2]	*Aralia racemosa*[2]	*Panax quinquefolius*[2]	*Panax trifolius*[2]
Analgesic	1	5	11	5
Blood & Heart	27	13	14	2
Gastrointestinal	5	7	6	2
General	2	3	10	-
Liver & Kidney	2	5	-	3
Nervous System	1	1	7	3
Other	1	-	1	2
Reproductive	2	11	4	2
Respiratory	9	20	6	3
Topical	19	16	12	4
Total[2]	74	78	65	21

[1] Usage category as defined by Jones (1999).

[2] Compiled from data published in Moerman (1998) and Arnason et al. (1981).

used tea of the whole plant to relieve colic, indigestion, gout, hepatitis, hives, rheumatism, and tuberculosis (Foster 1996)(Table 5.1).

Thin layer chromatographic analyses identified ginsenosides Ro, R_{b1}, R_{b2}, R_c, R_e, R_f, R_{g1} and R_{g2} in *P. trifolius* root (Lee and Der Mardersosian, 1981; Lui and Staba, 1980). HPLC analyses and verification with NMR spectroscopy (Lee and Der Marderosian, 1988a, 1988b) showed that kaempferol-3,7-dirhamnoside and kaempferol-3-gluco-7-rhamnoside occurred in all the plant parts, as did the ginsenosides R_o, R_{b1} and R_{b2}. Whereas R_{b3} was present in all the tissues except the roots, R_c was present in roots, leaves and flowers, R_d was present in the stems, leaves and flowers, and notoginsenoside-F_e was present in the stems and leaves.

The genus *Aralia* is in the most prominent North American genus of the ginseng family. The Araliaceous herbs, *Aralia nudicaulis* L. (Wild sarsaparilla) and *Aralia racemosa* L. (Spikenard, Life-of-man) have been used by Amerindians as tonics (Arnason et al., 1981). These grow in the hardwood forests of North America (Foster and Duke, 1990). *A. nudicaulis* rhizome infusion has been used by the Iroquois as a blood remedy and to treat colds; the Ojibwa used the rhizome as a poultice for boils and skin sores, in infusions as a blood purifier (Densmore, 1928), and as a remedy for cough, fainting and fits; rhizome decoctions were used for circulation and stoppage of menstruation, and rhizome powder was snuffed for nosebleed; and the Cree used a mash of rhizome for earaches (Arnason et al., 1981); and a beverage containing whole plant was used as a cooling medicine for the blood (Hussey, 1974) (Table 5.1).

A root infusion of *A. racemosa* has been used by the Iroquois, Micmac, Malecite and Ojibwa to treat colds and cough (Densmore, 1928); a root poultice was used by the Ojibwa for boils, fractures and strained and sprained muscles; a root decoction was used to treat cough, and for stoppage of menstruation; and the root was also used as a treatment for consumption, gonorrhoea, headache, kidney problems and female pain by the Malecite (Arnason et al., 1981) (Table 5.1).

A. racemosa roots do not contain alkaloids (MacCassady, 1965), but do contain oleanic acid glycoside (araloside) and β-sitosterol; the aerial tissue contains choline, chlorogenic and ursolic acids (Wróbel-Stermińska and Tomczyk, 1975). The diterpenes (-)kaur-16-en-19-oic acid and (-)pimara-8(14),15-dien-19-oic acid have been found in ethereal extracts from all tissues of *A. racemosa* (Mihashi, 1969; Shibata et al., 1967). A tincture of *A. racemosa*, along with 407 other tinctures of higher plants used in homoeopathic medicines, were tested for antituberculous activity; *A. racemosa* was shown to have some inhibitory effects on the growth of *Mycobacterium tuberculosis* (Grange and Davey, 1990).

In a phytochemical screening of 28 plants used in Micmac remedies, *A. nudicaulis* was shown to contain polyphenols and sterols in all plant tissues, triterpenes in the roots, leucoanthocyanin in the aerial tissue, and saponins in the stem; however, alkaloids, deoxysugars, flavonoids, unsaturated lactones and tannins were not detected (Chandler and Hooper, 1982). A subsequent elaboration of the screening (Hooper and Chandler, 1984) showed the sterols were largely β-sitosterol occurring in higher amounts in the roots, a smaller amount of stigmasterol occurring in higher amounts in the roots, and yet a smaller amount of campesterol

occurring throughout the plant. In addition, the triterpenes were α-amyrin and β-amyrin, which occurred in equal amounts throughout the plant. Larger amounts of unidentified triterpenes were also identified principally in the aerial parts of the plant. In a test for insecticidal properties, using products from 150 species and varieties of plants, an acetone extract of *A. nudicaulis* root was shown to kill *Culex quinquefasciatus* (mosquito) larvae (Hartzell and Wilcoxon, 1941). However, the test indicated that *A. nudicaulis* would not be an effective insecticide.

Since *A. nudicaulis*, *A. racemosa*, and *P. trifolius* have a history of medicinal use, often similar in ethnopharmacological uses to *P. quinquefolius* (Table 5.1), it was hypothesized that there is similarity in phytochemistry. Specifically, if it can be demonstrated that their active ingredients are ginsenosides, these common araliaceous plants could provide alternative sources for the demand in the marketplace for ginsenoside-containing drugs. Information on the phytochemical constituents of these species would be useful to breeders and biotechnologists contemplating "wide cross" manipulations of ginseng, or requiring genes for the biosynthesis of ginsenosides, or contemplating tissue culture. The harvest of these Araliaceous plants, instead of the wild crafting of ginseng, would conserve the endangered *P. quinquefolius*.

The objective of the present study was to determine common ginsenoside content in *A. nudicaulis*, *A. racemosa,* and *P. trifolius*.

5.2 Materials and Methods

5.2.1 Plant material

Plants were collected from sites in British Columbia, Manitoba, Ontario and Quebec. Voucher specimens for *A. nudicaulis*, *A. racemosa*, and *P. trifolius* have been deposited in the herbarium at the University of Ottawa (Table 5.2).

5.2.2 Extraction and analyses of ginsenosides

5.2.2.1 Extraction

The plants were dried on the laboratory benches at 20 - 22 °C for 4 - 6 weeks. Each plant part was then separately milled to no. 40 screen particle size (Thomas-Wiley laboratory mill, Philadelphia, PA). The ground plant material (0.01 – 1.0 g) was extracted twice with 20 % aqueous methanolic solution (30 ml) under reflux in a water bath at ~ 55 °C for 30 minutes each. The combined extract was evaporated (~ 40 °C) to dryness *in vacuo*, and the residue dissolved in 5 or 10 ml HPLC-grade methanol. This was then filtered using a 0.2 μm nylon filter (Chromatographic Specialties Inc., Brockville, ON) and 10 μl injected into the HPLC system.

5.2.2.2 Ginsenoside analysis

A Beckman High Pressure Liquid Chromatography (HPLC) system (Beckman Coulter Canada Inc., Mississauga, ON) was used for ginsenoside analysis. The system consists of a module 168 diode-array detector, a module 126 solvent delivery system, a module 502 autosampler, and a computer equipped with System Gold software.

Table 5.2 Collection data for populations of *Aralia nudicaulis*, *Aralia racemosa*, *Panax quinquefolius* and *Panax trifolius*.

Species	Voucher No.	Site (County/State)[1]	Number of individuals
Aralia nudicaulis	19516	Algoma, ON	3
	19517	Brandon, MB	2
	19518	Bristol, QC	2
	19519	Denholm, QC	1
	19520	Frontenac, ON	6
	19521	Haliburton, ON	1
	19522	Iberville, ON	3
	19523	Lanark, ON	4
	19524	Manitoulin Island, ON	2
	19525	Mt Robson, BC	5
	19526	Muskoka, ON	2
	19527	Ottawa-Carleton, ON	1
	19528	Pontiac, QC	2
	19529	Wentworth, QC	2
Aralia racemosa	19530	Denholm, QC	1
	19531	Frontenac, ON	3
	19532	Haliburton, ON	2
	19533	Iberville, ON	2

Species	Voucher No.	Site (County/State)[1]	Number of individuals
	19534	Lanark, ON	2
	19535	Muskoka, ON	5
	19536	Wentworth, QC	4
Panax quinquefolius	19501	Wisconsin	2
	19502	Lanark, ON	3
	19503	Frontenac, ON	5
	19504	Vermont	2
	19505	Maine	3
	19506	Haldiman-Norfolk, ON	6
	19507	Frontenac, ON	5
	19508	Frontenac, ON	3
	19509	Iberville, QC	5
	19510	Lennox and Addington, ON	4
Panax trifolius	19512	Algoma, ON	5
	19513	Frontenac, ON	5
	19514	Frontenac, ON	5
	19515	Leeds & Grenville, ON	5
	19516	Eastern Townships, QC	5

[1] Precise location by GPS is not provided to protect the wild populations of the uncommon *A. racemosa* and *P. trifolius*, and the endangered *P. quinquefolius*.

The ginsenosides R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2}, and R_d (from Dr. H Fong, University of Illinois at Chicago and Indofine Chemical Co., Somerville, NJ) were mixed together as pure standards at concentrations of 0.01 - 0.1 mg/ml in HPLC-grade methanol. Analyses based on peak area (AU) were performed, and the response factors (mg/ml/AU) were calculated and incorporated into the method. Response factors were continuously monitored and updated by bracketing the standard mixture between 6 – 10 samples.

The separation of the ginsenosides was achieved with reverse-phase Beckman ultrasphere C-18, 5 μm octadecylsilane, 250 x 4.6 mm column (Beckman Coulter Canada Inc., Mississauga, ON) connected to a LiChroCart LiChrospher 100 RP-18 4 x 4 mm, 5 μm guard column (EM Science, Cherry Hill, NJ). The mobile phase was water (A) and acetonitrile (B) at a constant composition of 21 % B from 0 - 20 min followed by a linear gradient to 42 % B from 20 - 60 min. The column was flushed and equilibrated after each analysis. The flow rate was 1.3 ml/min and the detector was set at 203 nm.

To confirm the identification of the ginsenosides in *A. nudicaulis* (Figure 5.1), *A. racemosa* (Figure 5.2) and *P. trifolius* (Figure 5.3), at least 3 samples (for each of the plant parts) from different populations were spiked with the standard mix, and these were run after their respective sample. In addition, purity checks were preformed by comparing the sample peaks of the particular plant with the standard peaks using on-line UV spectra. Comparisons with the UV spectra from *P. quinquefolius* peaks (Figure 5.4) were also done.

Figure 5.1 Chromatograms from HPLC analyses of ginsenosides in *Aralia nudicaulis* L. (Wild sarsaparilla) (a) leaves, (b) stem and (c) root.

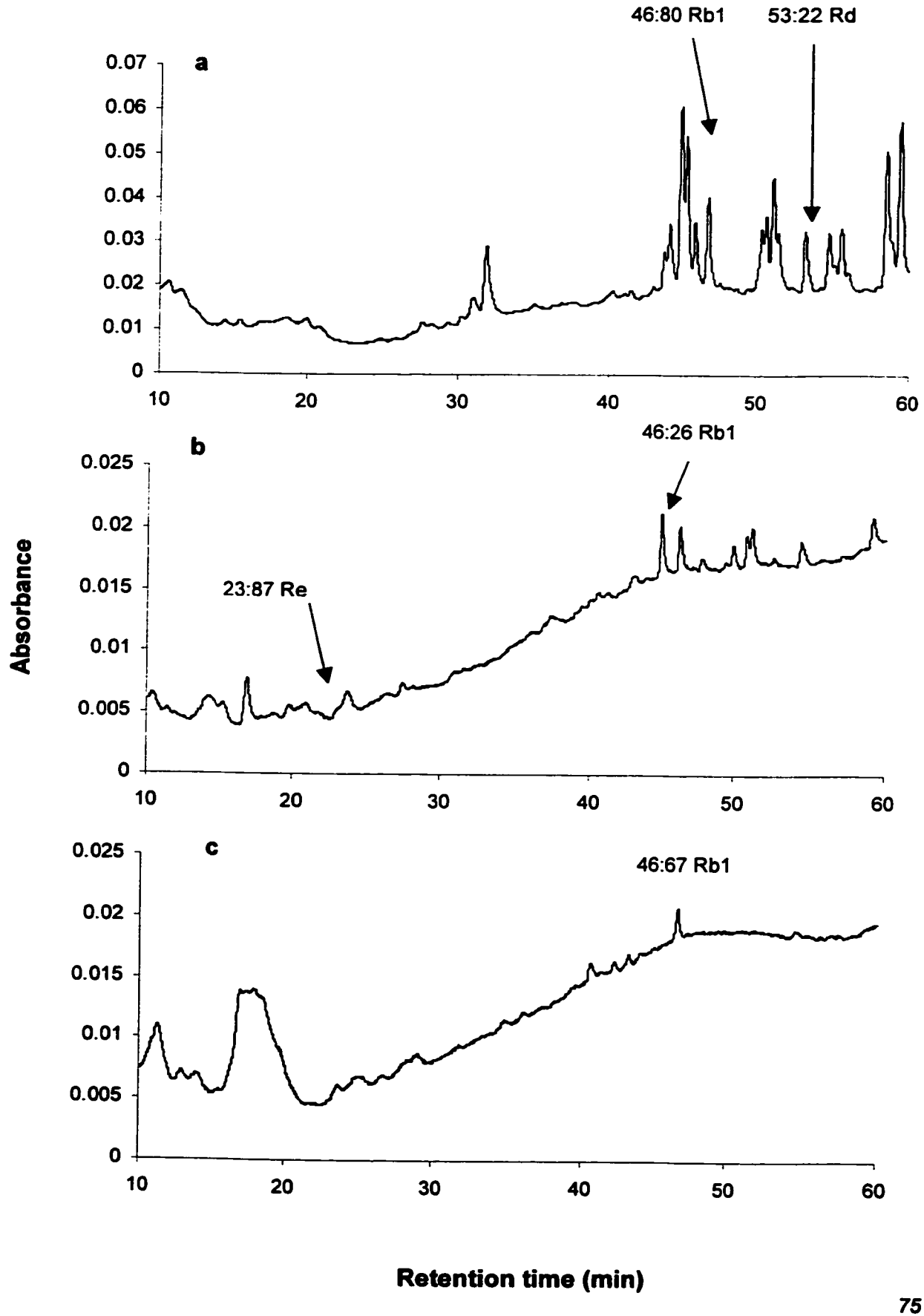

a
46:80 Rb1
53:22 Rd
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
b
46:26 Rb1
23:87 Re
0.025
0.02
0.015
0.01
0.005
0
c
46:67 Rb1
Absorbance
Retention time (min)

Figure 5.2 Chromatograms from HPLC analyses of ginsenosides in *Aralia racemosa* L. (Spikenard) (a) leaves, (b) stem and (c) root.

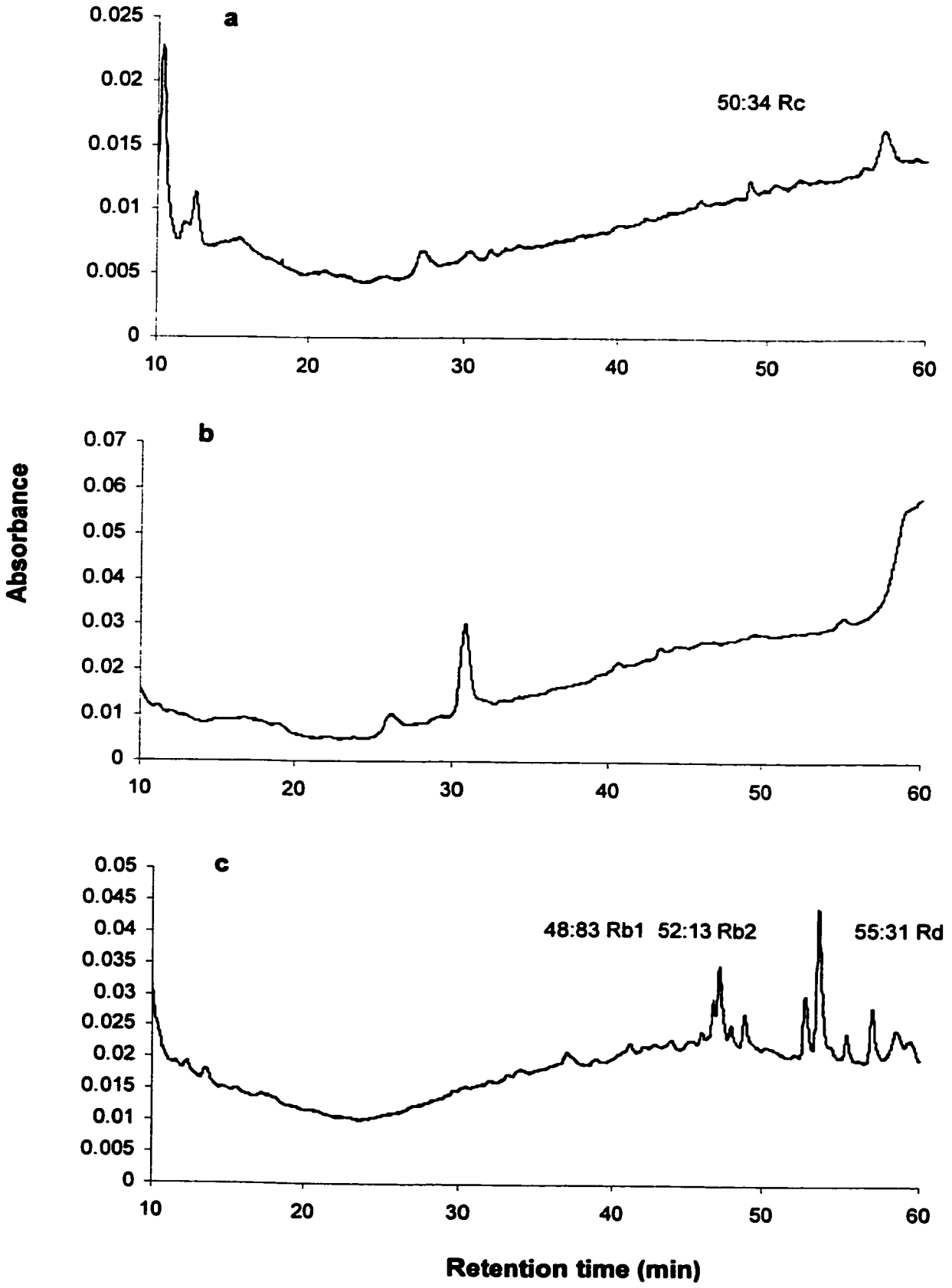

a
0.025
0.02
0.015
0.01
0.005
0
50:34 Rc
10
20
30
40
50
60
b
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
Absorbance
c
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
48:83 Rb1
52:13 Rb2
55:31 Rd
10
20
30
40
50
60
Retention time (min)

Figure 5.3 Chromatograms from HPLC analyses of ginsenosides in *Panax quinquefolius* L. (American ginseng) (a) leaves, (b) stem and (c) root.

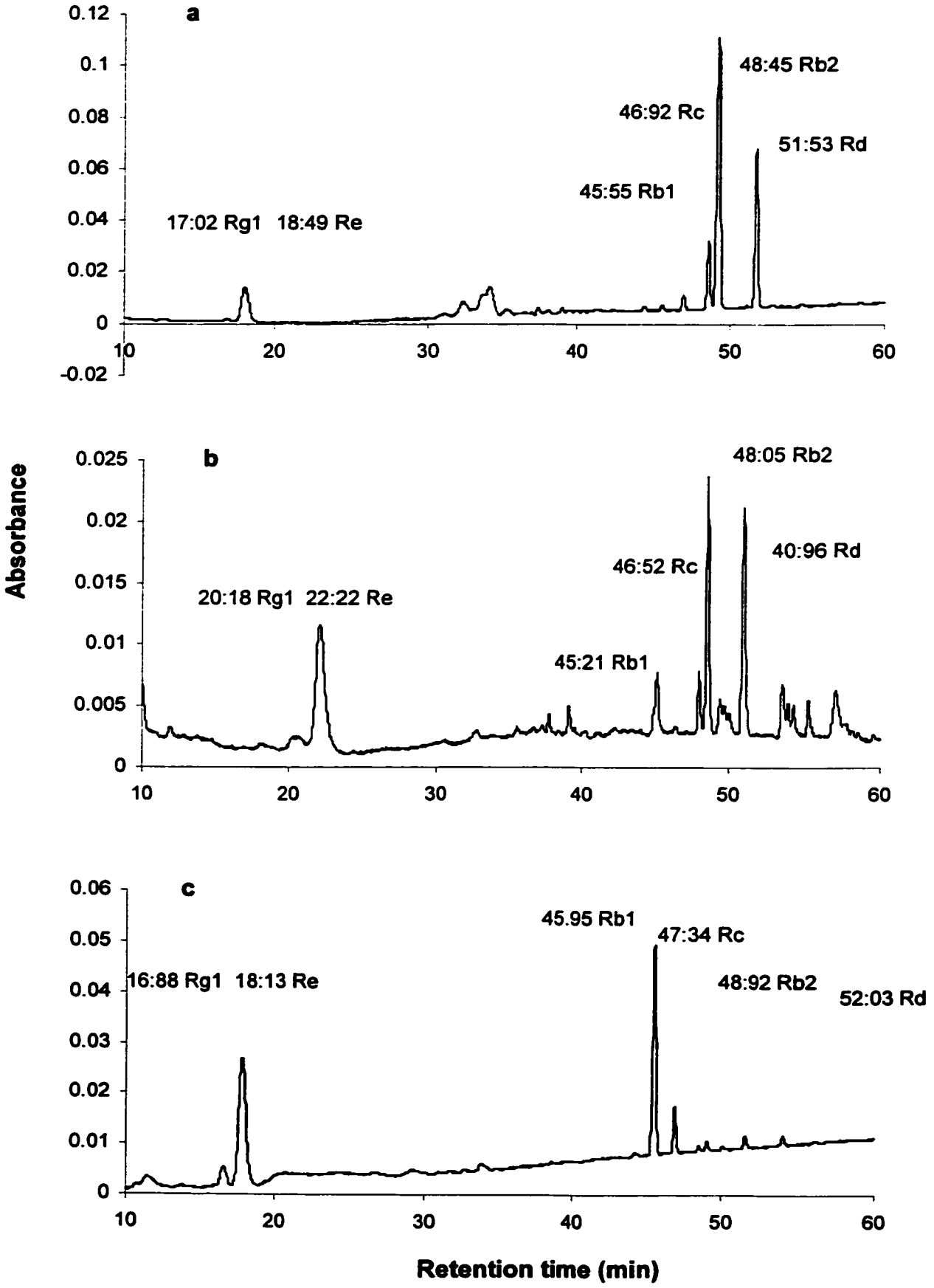
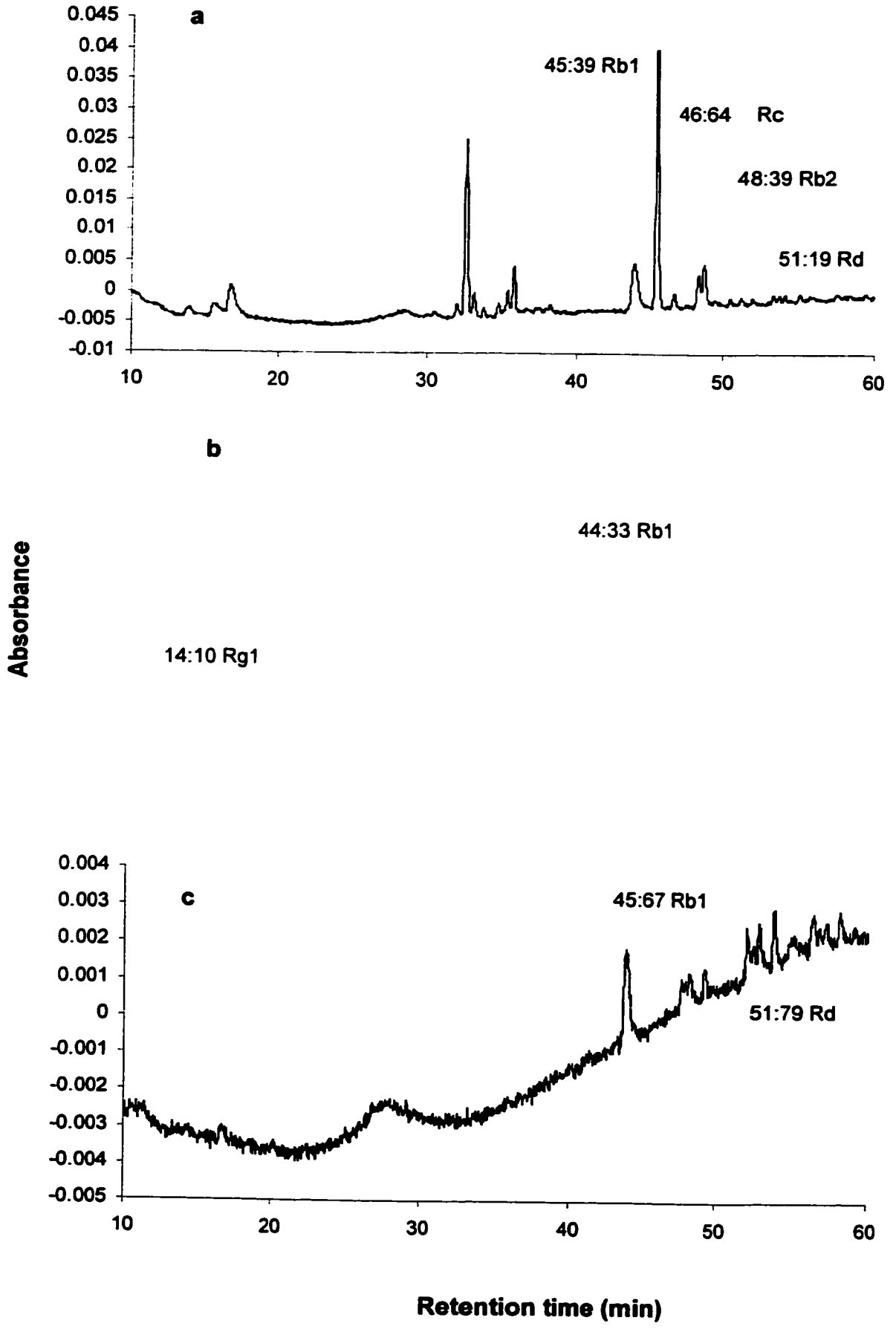

a
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
10
20
30
40
50
60
48:45 Rb2
46:92 Rc
51:53 Rd
45:55 Rb1
17:02 Rg1
18:49 Re
b
0.025
0.02
0.015
0.01
0.005
0
48:05 Rb2
40:96 Rd
46:52 Rc
20:18 Rg1
22:22 Re
45:21 Rb1
c
0.06
0.05
0.04
0.03
0.02
0.01
45.95 Rb1
47:34 Rc
16:88 Rg1
18:13 Re
48:92 Rb2
52:03 Rd
Absorbance
Retention time (min)

Figure 5.4 Chromatograms from HPLC analyses of ginsenosides in *Panax trifolius* L. (Dwarf ginseng) (a) leaves, (b) stem and (c) root.


a
45:39 Rb1
46:64 Rc
48:39 Rb2
51:19 Rd
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.01
10
20
30
40
50
60
b
44:33 Rb1
14:10 Rg1
Absorbance
c
45:67 Rb1
51:79 Rd
0.004
0.003
0.002
0.001
0
-0.001
-0.002
-0.003
-0.004
-0.005
10
20
30
40
50
60
Retention time (min)

All the solvents were HPLC grade: the acetonitrile and methanol were Omnisolv (EM Science, Gibbstown, NJ), and the distilled and de-ionized water was prepared using a Milli-Q Reagent Water System (Millipore Canada Ltd., Nepean, ON).

5.2.3 Statistical analyses

One-way analysis of variance (ANOVA) with Dunn's multiple comparison tests were performed using InStat 3.00 (GraphPad Software, San Diego, CA). All results were expressed as a mean of % w/w ± SEM and considered statistically significant if $P < 0.05$.

5.3 Results

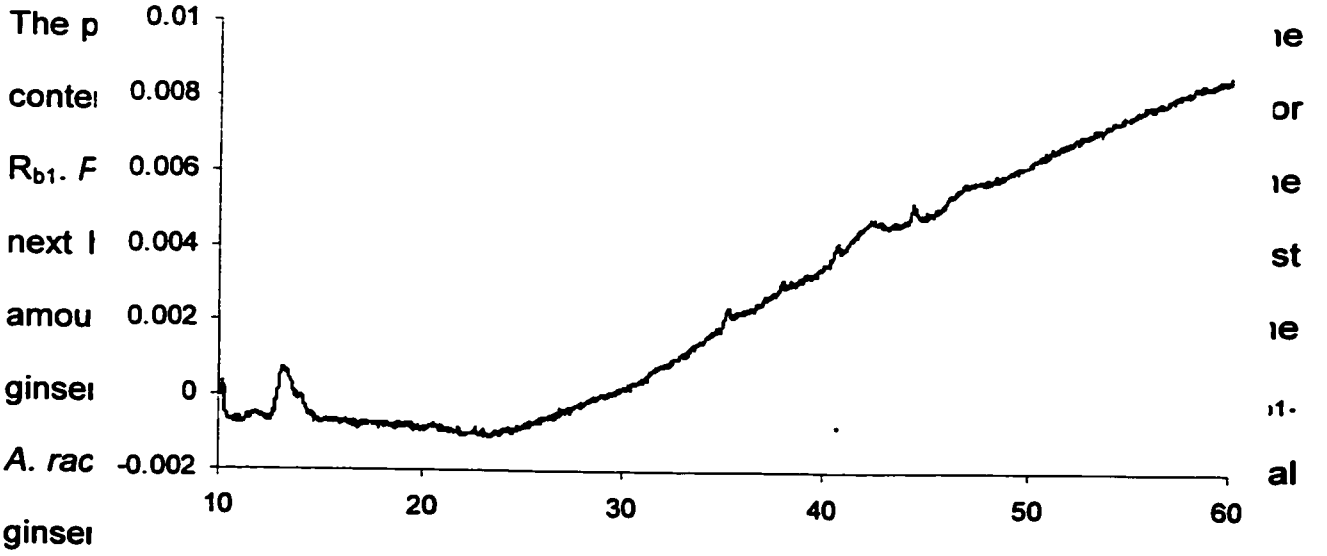


Table 5.4 shows the ginsenoside content in the stems of Canadian Araliaceae. The range in total ginsenoside content was 0.07 - 1.62 %.

Table 5.3 Ginsenoside content in the leaves of *Aralia nudicaulis*, *Aralia racemosa*, *Panax quinquefolius* and *Panax trifolius*.

Species	Ginsenoside Content (% w/w)[1]							
	R_{g1}	R_e	R_f	R_{b1}	R_c	R_{b2}	R_d	Total
A. nudicaulis	0.02[a]	0.01[a]	0.01[a]	0.21[a]	0.03[a]	0.03[a]	0.10[a]	0.44[a]
	(0.01)	(0.00)	(0.00)	(0.04)	(0.01)	(0.00)	(0.02)	(0.06)
A. racemosa	0.00[a]	0.00[a]	0.00[a]	0.00[b]	0.00[b]	0.01[a]	0.00[a]	0.02[a]
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.01)
P. quinquefolius	0.14[b]	0.93[b]	0.02[a]	0.17[a]	0.18[c]	1.04[b]	1.08[b]	3.35[b]
	(0.03)	(0.14)	(0.01)	(0.05)	(0.03)	(0.15)	(0.19)	(0.48)
P. trifolius	0.06[a]	0.05[a]	0.00[a]	1.59[c]	0.04[ab]	0.20[b]	0.06[b]	2.00[b]
	(0.04)	(0.02)	(0.00)	(0.20)	(0.01)	(0.05)	(0.01)	(0.24)

[1] The results are means of percentages based on the dry weight of the plant part. The values in brackets are SEM. For each column, different letters (a - c) following the mean indicate significant differences based on Dunn's test ($P < 0.05$). There were 36 samples for *A. nudicaulis*, 9 for *A. racemosa*, 19 for *P. quinquefolius* and 25 for *P. trifolius*.

Table 5.4 Ginsenoside content in the stems of *Aralia nudicaulis*, *Aralia racemosa*, *Panax quinquefolius* and *Panax trifolius*.

Species	Ginsenoside Content (% w/w)[1]							
	R_{g1}	R_e	R_f	R_{b1}	R_c	R_{b2}	R_d	Total
A. nudicaulis	0.00^a	0.01^a	0.00	0.03^a	0.01	0.01^a	0.00^a	0.07^a
	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.01)
A. racemosa	0.00^a	0.00^a	0.00	0.18^{ab}	0.01	0.00^a	0.11^{ab}	0.34^{ab}
	(0.00)	(0.00)	(0.00)	(0.15)	(0.01)	(0.00)	(0.09)	(0.24)
P. quinquefolius	0.12^b	0.52^b	0.00	0.11^b	0.05	0.36^b	0.46^{bc}	1.62^c
	(0.05)	(0.10)	(0.00)	(0.04)	(0.04)	(0.17)	(0.22)	(0.55)
P. trifolius	0.02^b	0.00^a	0.00	0.10^b	0.02	0.05^a	0.04^{ab}	0.23^b
	(0.01)	(0.00)	(0.00)	(0.03)	(0.01)	(0.03)	(0.02)	(0.07)

[1] The results are means of percentages based on the dry weight of the plant part. The values in brackets are SEM. For each column, different letters (a - c) following the mean indicate significant differences based on Dunn's test ($P < 0.05$). There were 37 samples for *A. nudicaulis*, 9 for *A. racemosa*, 19 for *P. quinquefolius* and 25 for *P. trifolius*.

P. quinquefolius had the highest amount of ginsenosides, then *A. racemosa*, and then *P. trifolius*. *A. nudicaulis* had the lowest amount of ginsenoside content. In *A. nudicaulis*, R_{b1} was 0.03 % and this comprised almost half of the total ginsenoside content of the stem. In fact, this R_{b1} profile was observed for *A. racemosa* and *P. trifolius* as well.

Table 5.5 compares the ginsenoside content of roots of the Canadian Araliaceae. Again, the highest amount of ginsenoside content was in *P. quinquefolius*: 5.78 % total ginsenoside content. *A. racemosa* had the next largest amount at 0.26 % due to a relatively high amount of R_d. Both *P. trifolius* and *A. nudicaulis* had small amounts of ginsenosides: 0.08 and 0.03 % respectively, in each case R_{b1} comprised almost half the total ginsenosides observed.

Both *A. nudicaulis* and *P. trifolius* had the greatest amount of ginsenosides in the leaves, whereas *A. racemosa* had the greatest amount in the stem. R_{b1} contributed to these high amounts. In these *Aralia*, the quantity of ginsenosides tended towards the micrograms per gram range compared to the milligrams per gram range of ginsenosides found in *P. quinquefolius*.

5.4 Discussion

This is the first report of the presence of ginsenosides in *A. nudicaulis* and *A. racemosa*; of the quantitative levels of the ginsenosides in the stem of *P. quinquefolius*; of the quantitative levels of the ginsenosides R_{g1} and R_e in the leaves, R_{g1} and R_c in the stem, and of R_d in the roots of *P. trifolius*.

Table 5.5 Ginsenoside content in the roots of *Aralia nudicaulis, Aralia racemosa, Panax quinquefolius* and *Panax trifolius*.

Species	Ginsenoside Content (% w/w)[1]							
	R_{g1}	R_e	R_f	R_{b1}	R_c	R_{b2}	R_d	Total
A. nudicaulis	0.00[a]	0.00[a]	0.00	0.01[a]	0.01[a]	0.00[a]	0.00[a]	0.03[a]
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)
A. racemosa	0.00[b]	0.01[a]	0.00	0.01[ab]	0.01[a]	0.07[a]	0.16[a]	0.26[a]
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.06)	(0.15)	(0.20)
P. quinquefolius	0.94[a]	1.42[b]	0.00	2.81[c]	0.42[b]	0.09[b]	0.29[b]	5.78[b]
	(0.24)	(0.17)	(0.00)	(0.36)	(0.06)	(0.01)	(0.08)	(0.59)
P. trifolius	0.00[b]	0.00[a]	0.00	0.03[b]	0.01[a]	0.01[a]	0.01[a]	0.08[a]
	(0.00)	(0.00)	(0.00)	(0.02)	(0.01)	(0.01)	(0.00)	(0.04)

[1] The results are means of percentages based on the dry weight of the plant part. The values in brackets are SEM. For each column, different letters (a - c) following the mean indicate significant differences based on Dunn's test ($P < 0.05$). There were 35 samples for *A. nudicaulis*, 13 for *A. racemosa*, 38 for *P. quinquefolius* and 38 for *P. trifolius*.

The results on the ginsenoside content of the leaves are similar to the results of Li et al. (1996) on cultivated *P. quinquefolius*. Although ginsenosides are reported in the stem (Chen et al., 1981), there has been no quantification of these ginsenosides in this plant part. The ginsenoside content results for the roots of *P. quinquefolius* are consistent with other reported results (Li et al., 1996; Court et al., 1996; Smith et al., 1996).

The Araliaceous plants *A. nudicaulis*, *A. racemosa*, and *P. trifolius* have significant ginsenoside content; but compared to *P. quinquefolius*, the quantities are much lower. *P. trifolius* leaves are the exception: the percentage of ginsenoside content is comparable to that found in the leaves of *P. quinquefolius*.

Other studies have shown that these Araliaceous species share other similarities in their phytochemistry. Kaempferol-3,7-dirhamnoside and kaempferol-3-gluco-7-rhamnoside (Lee and Der Marderosian, 1988a, 1988b) are common to *P. quinquefolius* and *P. trifolius*, and β-sitosterol occurs in both *A. nudicaulis* and *A. racemosa* (Hooper and Chandler, 1984; Wróbel-Stermińska and Tomczyk, 1975). Campesterol, stigmasterol and sitosterol have also been identified in *P. ginseng* roots (Chung, 1974). Polyacetylenes are also found in all four of these plants. In *P. ginseng*, they comprise part of the 1% lipid soluble compounds (Nah, 1997). All the components are structurally related: they are either C_{17}- or C_{18}-polyacetylenes derived from fatty acids with a characteristic terminal saturated aliphatic C_7H_{15} moiety for the C_{17}-compounds and a 3-hydroxy(or 3-oxo)hept-1-ene-4,6-diyne moiety for the C_{18}-compounds (Hansen and Boll, 1986). Table 1.1 lists the polyacetylenes investigated in the araliaceous members of the ginseng family.

Hansen and Boll (1986) reported antifungal activity from falcarinone, falcarinol and falcarindiol. In subsequent studies, the polyynes of *P. ginseng* demonstrated growth inhibition against Yoshida sarcoma cells (Fujimoto and Satoh, 1987) and cytotoxicity against numerous human tumour cell lines (Matsunaga et al., 1994; Matsunaga et al., 1990). *P quinquefolius* polyacetylenes were shown to completely inhibit the growth of leukaemia cells (Fujimoto et al., 1991).

In more recent studies, a close phylogenetic relationship detected by using sequences of the internal transcribed spacers and the 5.8S coding region of nuclear ribosomal DNA between *Aralia* and *Panax* was shown (Wen and Zimmer, 1996). This research also showed that *P. trifolius* was more closely related to *P. quinquefolius* than the members from the genus *Aralia,* even though *P. trifolius* was shown to be particularly divergent from other members of its genus.

Ginsenosides may not be the main active principles associated with many of the treatments listed for *Aralia* in Table 5.1. Since roots were most commonly used in traditional medicine (Moerman, 1998; Arnason et al., 1981) and I observed that ginsenosides were primarily present in the aerial parts, then we might expect the bioactivity from other compounds. Nevertheless, these species can be important ginseng germplasm resources.

The leaves of *P. trifolius* and *A. nudicaulis* may be useful sources for R_{b1}. This ginsenoside has been shown to stimulate the cholinergic system (Salim et al., 1997; Benishin et al., 1991) and is thus implicated in mediating learning and memory processes; it also has an antioxidative protective effect against nephrosis (Lim et al.,

1998). The metabolized products of R_{b1}, R_{b2} and Rc, named M1, induced melanoma cell death by regulating apoptosis-related proteins (Wakabayahi et al., 1998).

The use of *A. nudicaulis*, *A. racemosa* and *P. trifolius* would conserve the endangered *P. quinquefolius*. *A. nudicaulis* in particular is abundant in the wild, and although *P. trifolius* is not as common, the harvest of the leaves would protect its populations.

An alternative to obtaining ginsenosides from wild or cultivated plants would be a biotechnological approach. *In vitro* cultures have been successful in producing ginsenosides from various tissues of *P. ginseng*, including leaves (Furuya, 1988; Choi et al., 1982), stems (Furuya, 1988; Jhang et al., 1974), and roots (Linsefors et al., 1989; Furuya, 1988). Callus cultures from *P. quinquefolius* roots have also successfully produced various ginsenosides (Mathur et al., 1999; Zhong et al., 1996; Mathur et al., 1994). Both polysaccharide and ginsenoside production were significantly improved in *P. notoginseng* root cultures (Zhong and Wang, 1996). In another study, hairy root cultures from a hybrid of *P. ginseng* and *P. quinquefolius* produced greater amounts of ginsenosides than either parental species (Washida et al., 1998). These studies indicate that industrial production of ginseng's pharmacologically active metabolites, the saponins or the polysaccharides, via plant cell culture technology may be the most cost-effective alternative to the long and laborious cultivation of ginseng. Tissue cultures of *P. trifolius* or *A. nudicaulis* leaves or *A. racemosa* stems may also provide such alternative ginsenoside sources.

The following chapter has been accepted for publication by Phytomedicine, an international journal of phytotherapy and phytopharmacology. The article is entitled "Extractable polysaccharides of *Panax quinquefolius* L. (North American ginseng) root stimulate TNFα production by alveolar macrophages". The authors are V.A. Assinewe, J.T. Arnason, A. Aubry, J. Mullin, and I. Lemaire.

CHAPTER 6

THE IMMUNOPHARMACOLOGY OF *PANAX QUINQUEFOLIUS*

6.1 Introduction

Asian ginseng (*Panax ginseng* C.A. Meyer, Araliaceae) is known for its anti-stress and adaptogenic properties (Liu and Xiao, 1992), and for its pharmacological actions including cardioprotection (Maffei Facino et al., 1999), vasorelaxation (Rimar et al., 1996) and stimulation of the central nervous system (Liu and Xiao, 1992). One of the major effects of *P. ginseng* is the enhancement of host resistance against infection, both in experimental animals (Song et al., 1997) and in humans (Scaglione et al., 1994). This effect is most likely due to the stimulation of innate or non-specific immunity. *P. ginseng* stimulates non-specific immune response, notably by activating various macrophage functions including phagocytosis (Tomoda et al., 1994), intracellular killing and anti-*Candida* activity (Akagawa et al., 1996; Scaglione et al., 1994), and production of various cytokines such as interleukin 1α (IL-1α)(Kim et al., 1998), interleukin 8 (IL-8)(Sonoda et al., 1998) and interleukin 6 (IL-6)(Shin et al., 1998).

American ginseng (*Panax quinquefolius* L., Araliaceae), a closely related species originating in deciduous forest habitats of eastern North America, is a distinct medicinal plant in Asian traditions (Foster, 1996). *P. quinquefolius* has been used in traditional medicine by First Nation peoples to decrease fever, stomach pain and haemorrhage (Arnason et al., 1981). In recent studies, *P. quinquefolius* has

been shown to exhibit oestrogen-like activity (Duda et al., 1996), and to exert beneficial effects on the central nervous system (Lewis et al., 1999; Li et al., 1999b; Yuan et al., 1998) and the cardiovascular system (Li et al., 1999a). *P. quinquefolius* may produce effects similar to *P. ginseng,* and both are consumed for general well being (Eissenberg et al., 1993). However, relatively few studies have been performed with American ginseng, and its effects as an immunomodulator are at present unknown.

In this study, I investigated *P. quinquefolius*' immunostimulating activity in *vitro* by testing the ability of alveolar macrophages (AM) to produce tumour necrosis factor alpha (TNFα) in response to different extracts of *P. quinquefolius* root. TNFα is an important cytokine produced by activated macrophages during the inflammatory response (Beutler, 1999), and as part of host defence mechanisms against tumour (Srividya et al., 2000) and infection (Souto et al., 2000).

6.2 Materials and Methods

6.2.1 Animals

Male Wistar rats (250 - 300 g) were obtained from Charles River Canada Inc. (St-Constant, QC) where they were raised in a pathogen-free colony. The rats were shipped behind filter barriers, and upon arrival, housed in isolated, temperature-controlled quarters, in a horizontal laminar flow isolator (John Scientific Inc., Toronto, ON). They were given standard lab chow and water *ad libitum,* and were used within two weeks.

6.2.2 Plant materials and extraction

Four-year old roots of *P. quinquefolius* were obtained from Northern Lights Ginseng Farm (Quyon, QC), and dried at room temperature. A voucher specimen has been deposited in the herbarium at the University of Ottawa (Voucher No. 19511). Pure ginsenoside-R_{b1} (R_{b1}) was obtained from Indofine Chemical Co. (Somerville, NJ).

To prepare an aqueous root extract, a whole *P. quinquefolius* root was milled to No. 40 mesh (Thomas-Wiley laboratory mill, Philadelphia, PA) and 1 g of the ground root was sonicated at room temperature for 1 h in 10 ml water. The water-soluble fraction was filtered (Whatman No. 1, Clifton, NJ) and lyophilized to yield 365 mg of crude extract (Figure 6.1). To prepare a polyacetylene fraction, ~10 g of ground root was extracted in 100 ml of 95 % ethanol for 2 h on a shaker, and suction filtered (Whatman No. 1). The ethanol fraction was partitioned with 100 ml of hexane in a separatory funnel. The hexane fraction was reduced to 5 ml in a rotoevaporator, and the UV spectrum was scanned in quartz cuvettes in a Beckman double beam spectrophotometer with hexane as a blank. To prepare a ginsenoside-rich fraction, ~1 g of the ground *P. quinquefolius* root was extracted (Figure 6.2) in 200 ml methanol (MeOH) at room temperature for 24 h to remove soluble ginsenosides. Methanol extraction was performed twice, and the methanol-soluble fractions were combined, filtered (Whatman No. 1) then rotoevaporated to yield a total ginsenoside-enriched fraction (432 mg). The plant residue obtained from the methanol-insoluble fraction was further extracted twice in 200 ml hot water (95 °C) to remove soluble cell wall polysaccharides, and the combined water-soluble fractions were suction-

Figure 6.1 Extraction scheme of the aqueous extract from *Panax quinquefolius* root.

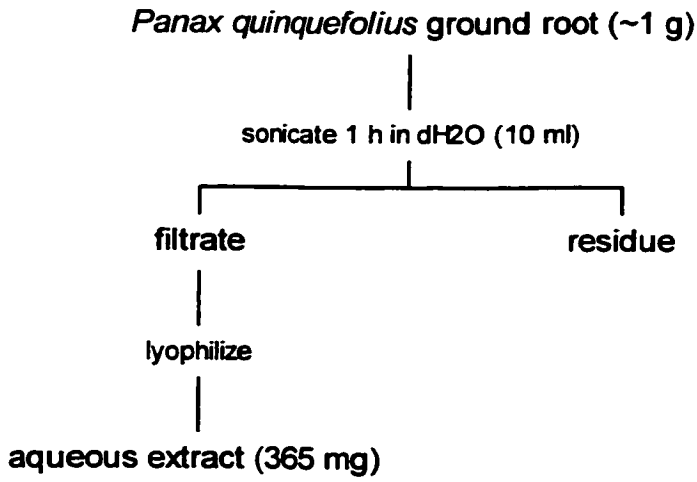
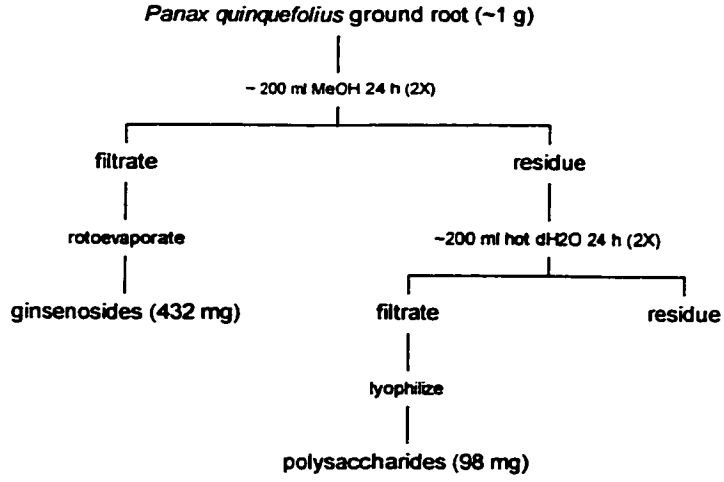

Panax quinquefolius ground root (~1 g)
sonicate 1 h in dH2O (10 ml)
filtrate
residue
lyophilize
aqueous extract (365 mg)

Figure 6.2 Extraction scheme for ginsenoside and extractable-polysaccharide fractions from *Panax quinquefolius* root.


Panax quinquefolius ground root (~1 g)
~ 200 ml MeOH 24 h (2X)
filtrate
residue
rotoevaporate
~200 ml hot dH2O 24 h (2X)
ginsenosides (432 mg)
filtrate
residue
lyophilize
polysaccharides (98 mg)

filtered (Whatman No. 1) and lyophilized to yield total extractable polysaccharides (98 mg). These extracts, as well as the R_{b1}, were resolubilized in water and diluted at appropriate concentrations in culture medium to stimulate the cells.

6.2.3 Ginsenoside analysis

A Beckman reverse-phase High Pressure Liquid Chromatography (HPLC) system was used for ginsenoside analyses. The system consists of a module 168 diode-array detector, a module 126 solvent delivery system, a module 502 autosampler, and a computer equipped with System Gold software. The ginsenosides R_{g1}, R_e, R_f, R_{b1}, R_c, R_{b2}, and R_d (Indofine Chemical Co., Somerville, NJ) were mixed together as pure standards at concentrations of 0.01 - 0.1 mg/ml in MeOH. Analyses based on peak area (AU) were performed and the response factors (mg/ml/AU) calculated and incorporated into the method. Response factors were continuously monitored and updated by bracketing the standard mixture between 6 – 10 samples. The separation of the ginsenosides was achieved with a reverse-phase Beckman ultrasphere C-18, 5 μm octadecylsilane, 250 x 4.6 mm column (Beckman Coulter Canada Inc., Mississauga, ON) connected to a LiChroCart LiChrospher 100 RP-18 4 x 4 mm, 5 μm guard column (EM Science, Cherry Hill, NJ). The mobile phase was water (A) and acetonitrile (B) at a constant composition of 21 % B from 0 - 20 min followed by a linear gradient to 42 % B from 20 - 60 min. The column was flushed and equilibrated after each analysis. The flow rate was 1.6 ml/min, and the detector was set at 203 nm. The residue from the MeOH extraction was dissolved in

10 ml MeOH, then filtered using a 0.2 μm nylon filter (Chromatographic Specialties Inc., Brockville, ON) and 5 μl injected into the HPLC system.

All solvents used in the extractions and HPLC analyses were HPLC grade (Omnisolv; EM Science, Gibbstown, NJ), and the water was distilled and de-ionized (Milli-Q Reagent Water System; Millipore Canada Ltd., Nepean, ON).

6.2.4 Analysis of crude polysaccharide extract

The extractable polysaccharide extract (50 mg) was hydrolysed in hot 1 M sulphuric acid (H_2SO_4)(3 ml, 97 °C) for 5 h with mixing every 30 min. After cooling, 0.5 ml of the extract was mixed with 1.5 ml water, filtered, and then analysed by HPLC. The neutral monosaccharides were separated on a DIONEX 4 x 250 mm PA-10 column fitted with a 4 x 50 mm PA-10 guard column. The HPLC system included Waters Millennium 32 software, a Waters 626 pump and 600S controller, a Waters 717plus Autosampler, and a Waters 464 pulsed amperometric detector. The mobile phase consisted of 100 mM sodium hydroxide (A), 300 mM sodium hydroxide (B), and water (C) at a constant flow rate of 1 ml/min. The elution profile was 2 % A and 98 % C for the first 32 min, followed by a 6 min linear gradient decrease of A to 0 % and a concomitant increase of B from 0 - 100 %, and maintained for 22 min. An external standard contained fucose, arabinose, rhamnose, galactose, glucose, xylose and mannose. Hydrolysates from similar extractions but using 12 M H_2SO_4 were also obtained, and uronic acids were determined from this preparation (Englyst et al., 1992). These analyses were calibrated against galacturonic acid and the absorbance measured at 400 and 450 nm to determine the difference in absorbance

due to uronic acids.

Simple starch tests were conducted by placing extracts on a filter and staining the samples with an iodine reagent (0.66 % KI and 0.33 % I_2).

6.2.5 Isolation of alveolar macrophages and stimulation

Alveolar macrophages (AM) were recovered from normal rats by bronchoalveolar lavage (BAL) (Lemaire, 1991). The lungs were lavaged with seven 7 ml aliquots of sterile phosphate buffered saline (pH 7.4; Wisent, St. Bruno, QC), and BAL cells were obtained by centrifugation at 200 x *g* at 4 °C for 5 min. The cells were resuspended in RPM1 1640 medium (Wisent, St Bruno, QC) supplemented with 0.5 % dialysed fetal bovine serum (Wisent, St Bruno, QC), 0.005 % gentamycin (Scherring Canada Inc., Pinot Claire, QC) and 0.8 % HEPES (Sigma Chemical Co., St Louis, MO). Cells were counted in a haemocytometer chamber, and cell viability (>98 %) was determined by trypan blue exclusion. Differential counts of BAL cells made from cytocentrifuge smears stained with Wright-Giemsa indicated that greater than 98 % of the BAL cell population was of macrophage morphology. AM (2×10^5) were incubated in 200 µl of complete medium in the presence and absence of various extracts of *P. quinquefolius* root or R_{b1} at different concentrations (0.1 - 250 µg/ml) for 19 h at 37 °C in a humidified atmosphere containing 5 % CO_2. Lipopolysaccharide (LPS, 1 µg/ml) (*Escherichia coli* Ser1027:B8; Sigma) was used as a positive control. Following incubation, the culture supernatants were collected by centrifugation and frozen at – 80 °C until assayed for TNFα.

6.2.6 Tumour necrosis factor alpha assay

Immunoreactive TNFα was measured in culture supernatants with a solid phase enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) employing the multiple antibody sandwich principle. Ninety-six-well microtiter plates were coated with polyclonal antibody specific for TNFα and culture supernatant was added to each well. After washing away unbounded substances, the culture was then incubated with polyclonal anti-murine TNFα antibody conjugated to horseradish peroxidase. After washing to remove unbound antibody-enzyme reagent, tetramethylbenzidine was added as a peroxidase substrate. Absorbance was read at 450 nm using an automated Spectra Count microplate reader (Packard Instrument Co., Meridan, CT).

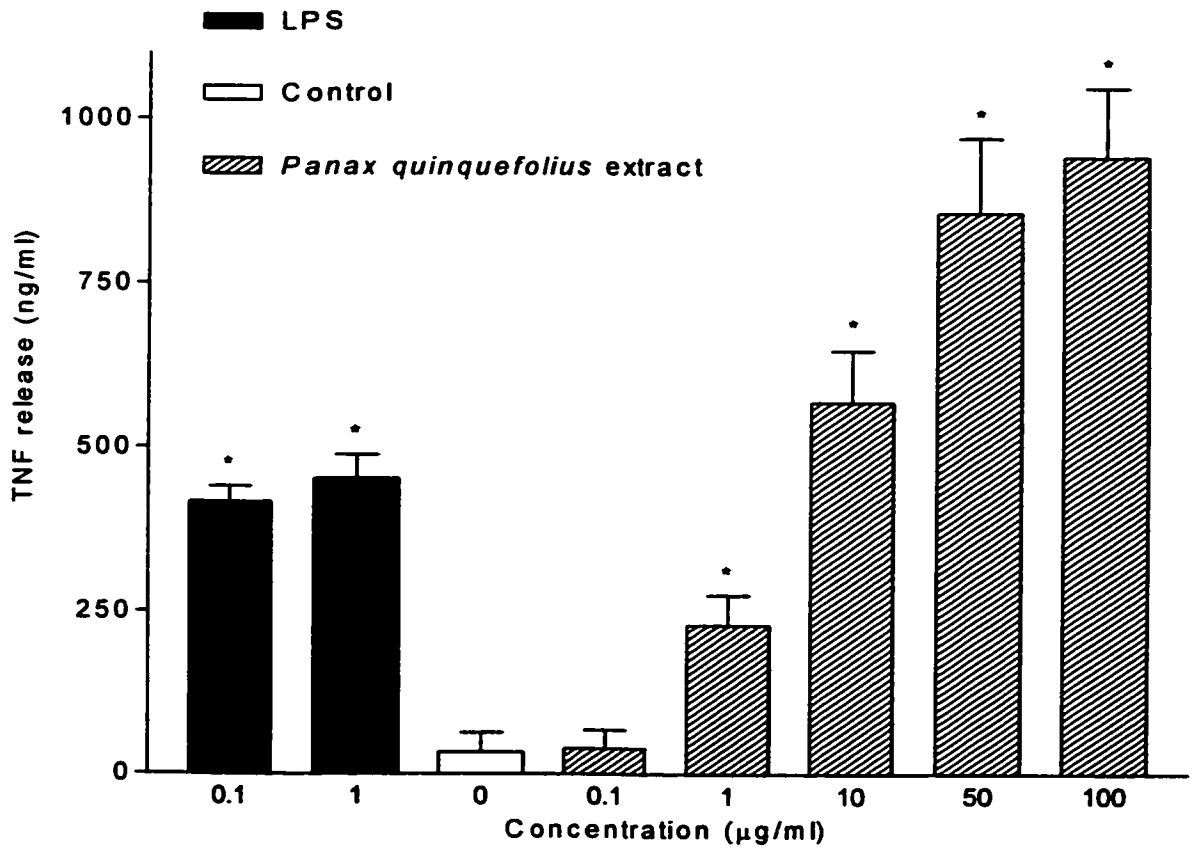
6.2.7 Statistical analysis

Each experiment was performed using AM from a different rat. Results are expressed as the mean of TNFα release (ng/ml) ± SEM from at least three separate experiments. One-way analyses of variance followed by Tukey's multiple range tests were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was set at $P < 0.05$.

6.3 Results and Discussion

Incubation of AM with the aqueous extract from *P. quinquefolius* root significantly stimulated the release of immunoreactive TNFα in the culture supernatants. As shown in Figure 6.3, stimulation was apparent at 1 μg/ml and

Figure 6.3 Effects of *Panax quinquefolius* aqueous root extract on TNFα production by rat alveolar macrophages. LPS (0.1 and 1 μg/ml) was the positive control. Results are the means of TNFα release (ng/ml) ± SEM of 6 separate experiments measured in triplicate. *Significant difference by Tukey test from the non-stimulated control ($P < 0.05$).


LPS
Control
Panax quinquefolius extract
TNF release (ng/ml)
1000
750
500
250
0
0.1
1
0
0.1
1
10
50
100
Concentration (µg/ml)

increased in a dose-dependent fashion, with maximal effect observed at 100 μg/ml, thus demonstrating the macrophage-activating potential of American ginseng. Higher concentrations of *P. quinquefolius* root extract (250 μg/ml (not shown)) retained significant stimulatory activity (albeit to a lower level), probably due to the presence of potential inhibitors in more concentrated extracts. In comparison with the bacterial antigen LPS, a known activator of AM (Chen et al., 1992), *P. quinquefolius* at concentrations of 0.1 - 1 μg/ml had stimulatory effects of lower magnitude on TNFα release. This is consistent with a beneficial immunostimulatory effect of *P. quinquefolius* in increasing cytokine production without the occurrence of significant toxicity, as has been observed with LPS. This bacterial endotoxin in sufficient concentrations can induce excessive production of inflammatory cytokines and endotoxic shock, a potentially fatal clinical condition.

I then attempted to identify the class of phytochemical responsible for TNFα stimulating activity. The hexane extract showed sharp multiple absorbance bands in the UV characteristic of ginseng polyacetylenes (Kwon et al., 1997; Bohlmann et al., 1973), but based on the extinction coefficients of these molecules, only trace amounts of these substances were detected (> 0.1 μg/g). Polyacetylenes are unstable, and are particularly prone to degradation in dried plant materials. The yield from our dried ginseng root was judged too low to contribute to biological activity, and was not subjected to bioassay.

A fraction enriched in ginsenosides, obtained by extraction in MeOH, contained a high level of the ginsenosides characteristic of *Panax* spp. (Table 6.1). The lack of an Rf peak and the high R_{b1}/R_{g1} ratio confirmed that authentic *P.*

quinquefolius and not *P. ginseng* roots were used. This fraction probably contained other components, but was free of high molecular weight polysaccharides that are insoluble in alcohols. As evidence of this, the characteristic iodine test for starch was negative.

A hot water extraction of the insoluble residue from the MeOH extraction yielded a soluble polysaccharide-enriched fraction. This extraction removed starch and extractable cell wall polysaccharides (pectins and hemicellulose, but not cellulose). After lyophilization the resulting fraction was a white cottony substance (~10 % of the dried root sample), which tested positive for starch with iodine.

To investigate the composition of the polysaccharides, acid hydrolysis analyses were undertaken. The 1 M H_2SO_4 hydrolyses revealed glucose, galactose, arabinose and rhamnose in the approximate ratio 85:8:6:1, and two other monosaccharides, fucose and mannose, in small amounts (Table 6.2). Hydrolyses with 12 M H_2SO_4 indicated the presence of approximately 9 % uronic acid in the extractable polysaccharide fraction. The acid hydrolyses did not investigate methoxy and acetoxy substitutions, and linkages were not identified. These results are consistent with the presence of starch and extractable neutral and/or acidic cell wall heteroglycans as found in other higher plants (Lien and Gao, 1990). The findings are similar to those of *P. ginseng* studies in which two acid polysaccharides, ginsenan PA and ginsenan PB, composed of arabinose, galactose, rhamnose, galacturonic and glucuronic acid were identified (Tomoda et al., 1994).

When the enriched fractions were incubated with AM (Figure 6.4) the extractable polysaccharides significantly stimulated TNFα release. The stimulation

Table 6.1 Ginsenoside content in different extracts of *Panax quinquefolius* root.

Extract	Ginsenoside content (% w/w)[1]						
	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d	Total
Aqueous extract[2]	0.00	0.25	0.97	0.84	0.03	0.94	3.22
Methanol fraction[3]	0.09	1.83	2.07	0.52	0.09	0.75	5.34
Water-soluble fraction[4]	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1] Content is based on the percentage of ginsenoside weight to the sample weight.

[2] Ground root sonicated in water.

[3] Enriched ginsenoside fraction.

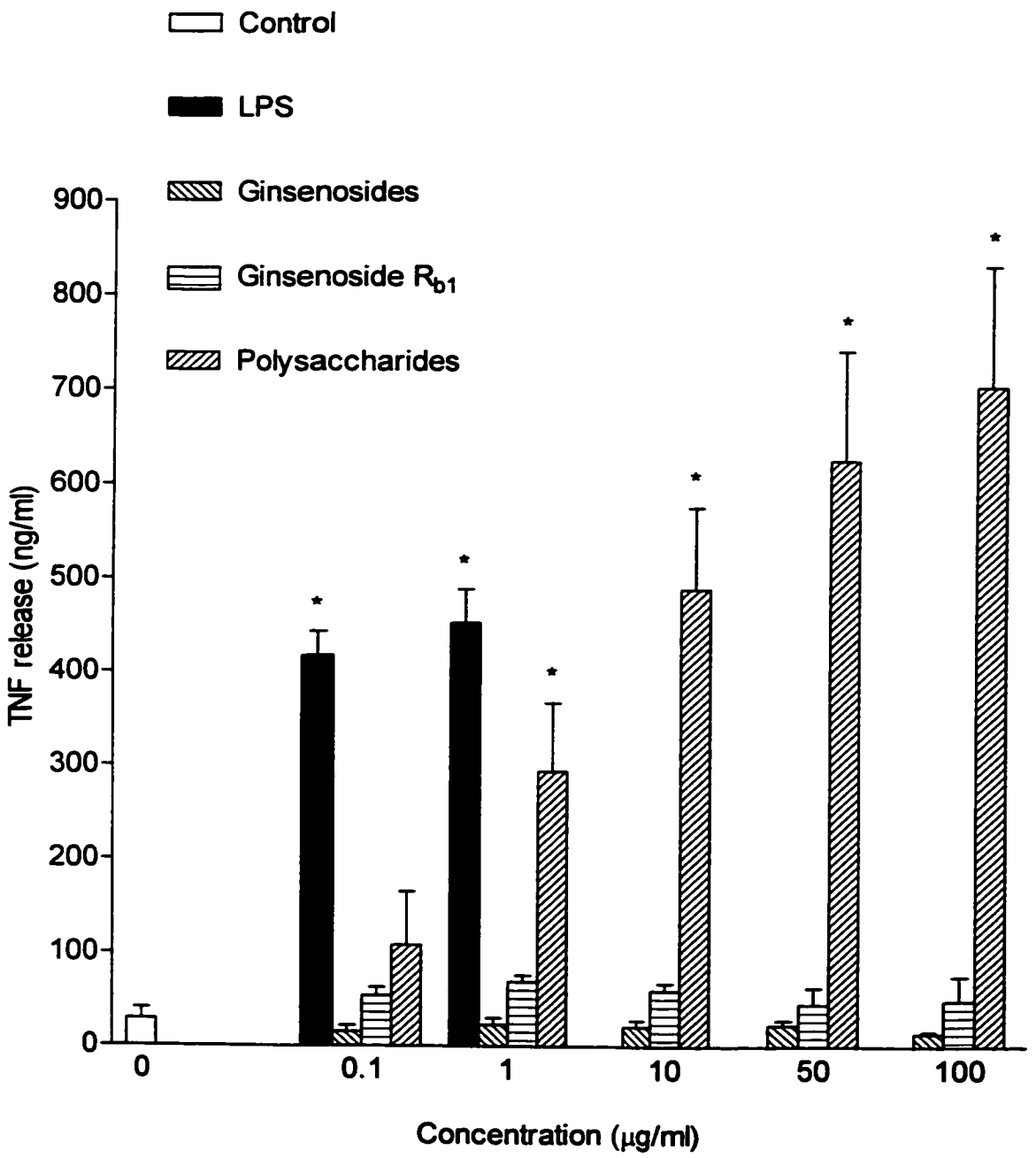
[4] Enriched polysaccharide fraction.

Table 6.2 Composition of crude polysaccharide fraction from 4-year old *Panax quinquefolius* root following acid hydrolyses[a].

Monosaccharides (%)					
Glucose	**Galactose**	**Arabinose**	**Fucose**	**Rhamnose**	**Mannose**
85.09	7.48	5.89	0.09	0.79	0.41

[a] From 1M H_2S0_4 hydrolysis of crude polysaccharide extract from 3 different roots.

Figure 6.4 Effects of various concentrations of ginsenoside and polysaccharide fractions from *Panax quinquefolius* root, and ginsenoside-R_{b1} on TNFα production by rat alveolar macrophages. The positive control was LPS (0.1 and 1 μg/ml). Results are the means of TNFα release (ng/ml) ± SEM of 5 separate experiments done in triplicate. *Significant difference from the non-stimulated control by Tukey test ($P < 0.05$).


Control
LPS
Ginsenosides
Ginsenoside R_{b1}
Polysaccharides
TNF release (ng/ml)
900
800
700
600
500
400
300
200
100
0
0
0.1
1
10
50
100
Concentration (μg/ml)

was dose-dependent (between 1 and 100 μg/ml) and comparable to that observed with aqueous root extracts. By contrast, the fraction containing total ginsenosides had little activity. To rule out the possibility that this ginsenoside preparation had lost activity during the fractionation procedure, I tested the effects of R_{b1}, which represents the major ginsenoside present in *P. quinquefolius* (Table 6.1). As was the case with total ginsenosides from *P. quinquefolius*, R_{b1} did not stimulate significantly TNFα production by rat AM. Therefore, the TNFα stimulating activity of *P. quinquefolius* root was associated almost exclusively with the soluble polysaccharide fraction. These observations are consistent with previous work, which showed that various polysaccharides from higher plants stimulate and modulate the immune system, notably through macrophage activation (Gao et al., 1996; Lien and Gao, 1990). Starch is inactive in these bioassays, and the immunomodulatory activity in other plants is normally associated with the soluble cell wall polysaccharides. In addition, polysaccharides from *P. ginseng* have been shown to be potent inducers of the monocyte and macrophage cytokines IL-8 (Sonoda et al., 1998), IL-6 (Shin et al., 1998) and IL-1α (Kim et al., 1998).

Further work is required to characterize the specific polysaccharide or combination of polysaccharides from *P. quinquefolius* that stimulate TNFα production. Previous studies have shown that in *P. ginseng*, the two acid polysaccharides ginsenan PA and PB have immunomodulating activity in carbon clearance tests (Tomoda et al., 1993b). Acidic arabinogalactans that induce peritoneal macrophages to secrete cytokines were isolated from *Echinacea purpurea* L. Moench, Asteraceae (Purple coneflower), and were particularly effective in

stimulating TNFα production (Luettig et al., 1989). In a more recent study, rhamnogalacturonan II (RG-II) from *P. ginseng* has been shown to be responsible for cytokine production (Shin et al., 1998).

In conclusion, this study demonstrates that *P. quinquefolius* shares with its Asian relative, *P. ginseng*, the ability to stimulate macrophage cytokine secretion, a crucial component of innate immunity. *P. quinquefolius* is a potent inducer of TNFα, an important cytokine in normal host response to infection (Souto et al., 2000; Srividya et al., 2000; Beutler, 1999), suggesting that it may augment the immune response. Whether *P. quinquefolius* also exerts other macrophage activating effects is currently under investigation. The observation that TNFα stimulation resides with the polysaccharide fraction further indicates that the macrophage-stimulating activity of *P. quinquefolius* preparations may be partially standardized according to their polysaccharide content.

CHAPTER 7

GENERAL CONCLUSIONS AND DISCUSSION

The goal in this research, which was to better characterize the phytochemistry and biological activity of ginseng germplasm resources, has been achieved. Scientific investigation of the immunopharmacology of *Panax quinquefolius* L. (American ginseng) provides new insight into the body of traditional knowledge accumulated over millennia by Aboriginal and other peoples.

7.1 Main conclusions

7.1.1 AOAC method validation for ginseng analysis

Fifteen laboratories from six different countries and regions, including our laboratory, participated in this collaborative study. The goal was to validate an HPLC method for the determination of the major ginsenosides R_{b1}, R_{b2}, Rc, Rd, Re, and R_{g1}, and to use it to estimate the ginsenoside content in extracts of *P. ginseng* and *P. quinquefolius* and their commercial products.

The values obtained for system suitability, intra-laboratory repeatability, and inter-laboratory reproducibility were generally consistent among the collaborators and within acceptable ranges, although there were exceptions.

Overall, there were only two outliers in the determination of individual ginsenosides in the four test samples. However, more than five outliers were found in the determination of R_{g1} and Re in one of the ginseng products. As a

consequence, the HPLC method was held not to be rigorous for the analysis of R_{g1} and Re in ginseng products at levels below 300 mg/kg.

Though the extraction method in this study yields slightly less ginsenosides than the extraction method used in the rest of my research (Chapters 3 – 6), the method is simpler and less time consuming. For this reason, the AOAC extraction and HPLC methods were used to determine ginsenoside content in subsequent analyses such as the determination of ginsenoside content in ginseng products used in Vuksan et al.'s diabetes clinical research (2001).

7.1.2 Processing methods for ginsenoside analysis

Processing methods clearly affect ginsenoside content. The highest levels of total ginsenoside content and the best quantities of R_{g1}, R_{b1} and R_d are achieved by drying at high temperature, whereas initial steam processing followed by drying at high temperature yields lower ginsenoside levels. In fact, my experience indicates that the longer the steaming process, the lower the levels in major ginsenosides resulting in an increase of the R_{b1} to R_{g1} ratio.

Ginsenoside-R_{b1} has been shown to be a central nervous system depressant, and to have antipsychotic, hypotensive, antistress, anti-inflammatory, gastro-intestinal motility activating, and antihaemolytic actions. In contrast, R_{g1} has been shown to stimulate the CNS, and have hypertensive, antifatigue and stress ulcer-aggravating actions (Hikino, 1991). These actions explain the use of R_{b1} as the 'yin' agent (cooling) and R_{g1} as the 'yang' agent (warming) in Traditional Chinese Medicine.

The contradictory physiological effects reported from ginseng (Hikino, 1991) may be the result of differences in processing methods of ginseng products. However, other compounds generated by the steaming process may also account for these pharmacological activities. Further immunopharmacological tests are required to compare the effects of red and white ginseng.

Although ginseng is primarily taken to prevent illness—as a tonic—in both Amerindian and Chinese traditional medicines, it is also used to treat illness. It is this use in therapeutic medicine that measures to ensure quality control and standardization of ginseng is especially important. The efficacy and safety of all herbs and phytomedicines taken concomitantly with prescription drugs should be available to all natural health products users.

7.1.3 Phytochemistry of *Panax quinquefolius*

This is the first report that characterizes the levels of naturally occurring ginsenosides through a significant survey of wild Canadian ginseng populations. Despite the limitations in sample size due to its endangered status, this study demonstrated that there is significant phytochemical variation in the major ginsenosides between wild populations of *P. quinquefolius*, and that age was not a factor that could be detected in this variation. A study using a larger number of populations and increased sample size may never be possible, given the endangered status of the wild populations of *P. quinquefolius* and the collection limits set by conservation authorities. The variation observed in populations suggests

that conservation efforts need to target specific populations as well as the species as a whole.

The study produced baseline data on authentic *P. quinquefolius,* which will provide an information resource to ginseng wild crafters and growers on total ginsenoside levels. This reference information is also important as a future benchmark for quality control studies such as the American Botanical Council's Ginseng Evaluation Program.

This work is the first to quantify the major ginsenosides in the stem. Contrary to previous data (Li et al., 1996), this study showed that ginsenoside-R_{b2} is a significant saponin in *P. quinquefolius* leaves. I also showed that there was no statistical difference in ginsenoside content between wild and cultivated *P. quinquefolius* roots of the same age.

This study was unable to address environmental and genetic effects because of the small sample size. Field studies will be required to explore these effects conclusively. Such a study was beyond the scale of my research, since 12 months of stratification are required to germinate the seeds and at least a 4-year growth period is required for the plant to reach maturity (Schluter and Punja, 2000; Proctor and Bailey, 1987; Lewis and Zenger, 1983). Field studies would provide information on superior genotypes under controlled growth conditions, and could help support genetic improvement of *P. quinquefolius* for commercial exploitation while protecting extant wild populations.

7.1.4 Phytochemistry of eastern Canadian Araliaceae

This is the first report of the presence of ginsenosides in *Aralia nudicaulis* L. (Wild sarsaparilla) and *Aralia racemosa* L. (Spikenard), including the first findings on quantitative levels of the major ginsenosides in the stem of *P. quinquefolius*, and the ginsenosides R_{g1} and R_e in the leaves, R_{g1} and R_c in the stem, and R_d in the roots of *Panax trifolius* L. (Dwarf ginseng).

With the exception of *P. trifolius*, the quantities of major ginsenosides are much lower, often measurable only in micrograms per gram, in *A. nudicaulis*, *A. racemosa*, and *P. trifolius,* compared to levels measurable in milligrams per gram in *P. quinquefolius*. Of note also is that ginsenosides were primarily present in the aerial parts of these Araliaceous herbs.

This study suggests that these Araliaceous plants may serve as a ginseng germplasm resource, providing ginsenosides through tissue cultures, or supporting the breeding of superior lines of *P. quinquefolius*. *P. trifolius*, for example, is more tolerant to low light and cold which may be an important economic benefit.

Further investigation is required on the best population sources of ginsenosides in these Araliaceous herbs. In addition, given the wide use of these plants as tonics and in the traditional medicine of Aboriginal peoples, particularly the two *Aralia* species, their immunopharmacological activity should by investigated.

7.1.5 Immunopharmacology of *Panax quinquefolius*

This study demonstrated the macrophage-activating potential of *P. quinquefolius*: macrophage activation is a crucial function in innate immunity.

Aqueous extracts from the root significantly stimulated the release of immunoreactive TNFα in culture supernatants.

To identify the class of phytochemical responsible for TNFα stimulating activity, crude fractions of the root were prepared and tested for their immunostimulating response. Polyacetylene content in the dried ginseng root was judged too low to contribute to biological activity, and was not subjected to bioassay. A ginsenoside-rich fraction obtained from a methanolic extraction of the root showed little activity, as did the pure ginsenoside-R_{b1}. However, the soluble polysaccharide-enriched fraction, which was extracted from the insoluble residue resulting from the methanol extraction, stimulated TNFα release to the same extent as the total root extract.

The crude extractable polysaccharide fractions were analysed, and these contained a high level of glucose, smaller levels of uronic acid, galactose, arabinose and rhamnose, and trace levels of fucose and mannose.

Identifying the *P. quinquefolius* constituents that enhance and inhibit TNFα release will explain the mode of action for this cytokine. *P. quinquefolius* could be a valuable therapeutic product for inflammatory and immunological diseases that are mediated by TNFα (Habtemariam, 2000).

We are currently investigating whether *P. quinquefolius* can potentiate other macrophage activities. Further work is required to look at other immune response effects as well as other pharmacological effects of *P. quinquefolius* and to identify the mechanism of action. Lee et al. (1997) recently showed that ginsenoside R_{g1}, a protopanaxatriol of *P. ginseng* and *P. quinquefolius*, acted as a functional ligand of glucocorticoid receptor. Bound glucocorticoid receptor, when complexed with

glucocorticoid response elements, regulates the transcription of target genes. However, Lewis et al. (1999) showed that it was not ginsenosides that were involved in the stimulation of nicotinic activity that occurred with the crude extracts of *P. quinquefolius* and *P. ginseng*. Since ageing in the brain is associated with the loss of nicotinic receptor binding, identifying the bioactive chemical constituent or constituents in *Panax* spp. would provide a therapeutic agent for age-related cognitive impairments.

The research on *Panax* spp. may lead to the standardization of ginseng products according to their pharmacological properties, and the development of cultures and breeding programs to produce superior levels of the bioactive constituents.

REFERENCES

Akagawa G, Abe S, Tansho S, Uchida K, Yamaguchi H. 1996. Protection of C3H/HE J mice from development of *Candida albicans* infection by oral administration of Juzen-taiho-to and its component, Ginseng radix: possible roles of macrophages in the host defense mechanisms. Immunopharmacology and Immunotoxicology 18(1):73-89.

Anderson RC, Fralish JS, Armstrong JE, Benjamin PK. 1993. The ecology and biology of Panax quinquefolium L. (Araliaceae) in Illinois. American Midland Naturalist 129(2):357-372.

Argus GW, Keddy CJ, White DJ. 1984. Rare species of Araliaceae. In: Argus GW, Pyryer KM, White DJ, Keddy CJ. (Eds.), Atlas of the Rare Vascular Plants of Ontario. Part 3. National Museum of Natural Sciences, National Museums of Canada, Ottawa ON.

Arnason T, Hebda RJ, Johns T. 1981. Use of plants for food and medicine by Native Peoples of eastern Canada. Canadian Journal of Botany 59(11):2189-2325.

Atopkina LN, Malinovskaya GV, Elyalov GB, Uvarova NI, Woerdenbag HJ, Koulman A, Pras N, Potier P. 1999. Cytotoxicity of natural ginseng glycosides and semisynthetic analogues. Planta Medica 65:30-34.

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(1):111-115.

Baldwin IT, Preston CA. 1999. The eco-physiological complexity of plant responses to insect herbivores. Planta 208:137-145.

Bazzaz FA, Chiariello NR, Coley PD, Pitelka LF. 1987. Allocating resources to reproduction and defense. BioScience 37(1):58-67.

Benishin CG, Lee R, Wang LCH, Liu HJ. 1991. Effects of Ginsenoside R_{b1} on central cholinergic metabolism. Pharmacology 42:223-229.

Beutler BA. 1999. The role of tumor necrosis factor in health and disease. The Journal of Rheumatology 26(Supp57):16-21.

Bohlmann F, Burkhardt T, Zdero C. 1973. Naturally Occurring Acetylenes. Academic Press, New York, NY.

Borchers AT, Keen CL, Stern JS, Gershwin ME. 2000. Inflammation and Native American medicine: the role of botanicals. American Journal of Clinical Nutrition 72(2):339-347.

Brevoort P. 1996. The US botanical market—an overview. HerbalGram 36:49-57.

Carlson AW. 1986. Ginseng: America's botanical drug connection to the Orient. Economic Botany 40(2):233-249.

Carpenter SG, Cottam G. 1982. Growth and reproduction of American ginseng (*Panax quinquefolius*) in Wisconsin, USA. Canadian Journal of Botany 60:2692-2696.

Catling PM, Spicer KW. 1995. Pollen vectors in an American ginseng (*Panax quinquefolius*) crop. Economic Botany 49(1):99-102.

Chandler RF, Hooper SN. 1982. Herbal remedies of the Maritime Indians: A preliminary screening. Part III. Journal of Ethnopharmacology 6:275-285.

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

Chen SE, Staba EJ, Taniyasu S, Kasai R, Tanaka O. 1981. Further study on dammarane-saponins of leaves and stems of American ginseng, Panax quinquefolium. Planta Medica 42:406-411.

Chen TY, Lei MG, Suzuki T, Morrison DC. 1992. Lipopolysaccharide receptors and signal transduction pathways in mononuclear phagocytes. Current Topics in Microbiology and Immunology 181:169-188.

Choi KJ, Lee KS, Ko SR, Jang JG, Park JD, Kim MW, Sung HS. 1988. Quality stability and antioxidant activity of Red ginseng stored for long periods. Proceedings of the 5th International Ginseng Symposium, Seoul, 1988.

Choi KT, Kim MW, Shin HS. 1982. Induction of callus and organ in tissue culture of ginseng (*Panax ginseng* CA Meyer). Korean Journal of Ginseng Science 6:162-167.

Chung BS. 1974. Studies on the constituents of Korean ginseng (1). On the constituents of Korean ginseng sterols. Korean Journal of Pharmacognosy 5:175-

Court WA, Reynolds LB, Hendel JG. 1996. Influence of root age on the concentration of ginsenosides on American ginseng (*Panax quinquefolium*). Canadian Journal of Plant Science 76:853-855.

Densmore F. 1928. Uses of Plants by the Chippewa Indians. Forty-fourth Annual Report of the Bureau of American Ethnology, 1926-1927. (Reproduced as the first Canadian edition in 1987 entitled Indian Use of Wild Plants for Crafts, Food, Medicine, and Charms. Iroqrafts: Ohsweken, ON).

Duda RB, Taback B, Kessel B, Dooley DD, Yang H, Marchiori J, Slomovic BM, Alvarez JG. 1996. pS2 expression induced by American ginseng in MCF-7 breast cancer cells. Annals of Surgical Oncology 3(6):515-520.

Duke JA. 1989. Ginseng: A Concise Handbook. Reference Publications Inc., Algonac, MI.

Eissenberg D.M., Kessler R.C., Foster C., Norlock F.E., Calkins D.R., Delbanco T.L. 1993. Unconventional medicine in the United States. New England Journal of Medicine 328: 246-252.

Englyst HN, Quigly ME, Hudson GJ, Cummings JH. 1992. Determination of dietary fibre as non-starch polysaccharides by gas chromatography. Analyst 117:1707-1714.

Erichsen-Brown C. 1979. Medicinal and Other Uses of North American Plants: A Historical Survey with Special Reference to The Eastern Indian Tribes. Dover Publications Inc., New York, NY.

Evans BL. 1985. Ginseng: root of Chinese-Canadian relations. Canadian Historical Review 66:1-26.

Fisher P. 1993. Growing ginseng in Ontario. Proceedings of the Ontario Horticultural Crops Conference, 116-121.

Fitzloff JF, Yat P, Lu ZZ, Awang DVC, Arnason JT, van Breeman RB, Hall T, Blumenthal M, Fong HHS. 1999. Perspectives on the quality assurance of ginseng products in North America. *In* Huh H, Choi Kj, Kim YC (Eds.) Advances in Ginseng Research: Proceedings of the 7th International Symposium on Ginseng. Seoul, Korea, September, 1998: Korean Society of Ginseng, 138-145.

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

Foster S, Duke JA. 1990. A Field Guide to Medicinal Plants: Eastern and Central North America. The Peterson Field Guide Series 40. Houghton Mifflin Co., New York, NY.

Fujimoto Y, Satoh M. 1987. Acetylenes from the callus of *Panax ginseng.* Phytochemistry 26(10):2850-2852.

Fujimoto Y, Satoh M, Takeuchi N, Kirisawa M. 1991. Cytotoxic acetylenes from *Panax quinquefolium.* Chemical and Pharmaceutical Bulletin 39(2):521-523.

Fulder S. 1980. The Root of Being. Ginseng and the Pharmacology of Harmony. Hutchinson, London, UK.

Furuya T. 1988. Saponins (Ginseng Saponins). Cell Culture and Somatic Cell Genetics of Plants 5:213-234.

Fushimi H, Komatsu K, Isobe M, Namba T. 1996. 18S ribosomal RNA gene sequences of three *Panax* species and the corresponding ginseng drugs. Biological & Pharmaceutical Bulletin 19(11):1530-1532.

Gao H, Wang F, Lien EJ, Trousdale MD. 1996. Immunostimulating polysaccharides from *Panax notoginseng*. Pharmaceutical Research 13(8):1196-1200.

Gao QP, Kiyohara H, Cyong JC, Yamada H. 1991. Chemical properties and anti-complementary activities of heteroglycans from the leaves of *Panax ginseng*. Planta Medica 57:132-136.

Gleason HA. 1968. The New Britton and Brown Illustrated Flora of the Northeastern United States and Adjacent Canada. Volume 2 The Choripetalous Dicotyledoneae. Hafner, New York, NY.

Grange JM, Davey RW. 1990. Detection of antituberculous activity in plant extracts. Journal of Applied Bacteriology 68:587-591.

GraphPad InStat version 3.05 for Windows 95. 1998. GraphPad Software Inc., San Diego, CA.

GraphPad Prism version 3.00 for Windows. 1999. GraphPad Software Inc., San Diego, CA.

W. Greuter W, McNeill J, Barrie FR, Burdet HM, Demoulin V, Filgueiras TS, Nicolson DH, Silva PC, Skog JE, Trehane P, Turland NJ, Hawksworth DL. 2000. International Code of Botanical Nomenclature (St Louis Code). Regnum Vegetabile 131. Koeltz Scientific Books, Königstein, Germany.

Habtemariam S. 2000. Natural inhibitors of tumour necrosis factor-α production, secretion and function. Planta Medica 66:303-313.

Hansen L, Boll PM. 1986. Polyacetylenes in Araliaceae: their chemistry, biosynthesis and biological significance. Phytochemistry 25(2):285-293.

Hardacre V. 1974. Woodland Nuggets of Gold: The Story of American Ginseng Cultivation. Holland House Press, Northville, MI.

Harlan JR, deWet JMJ. 1971. Toward a rational classification of cultivated plants. Taxonomy 20: 509-517.

Hartzell A, Wilcoxon F. 1941. A survey of plant products for insecticidal properties. Contributions from Boyce Thompson Institute 12:127-141.

Hikino H. 1991. Traditional remedies and modern assessment: the case of ginseng. In: Wijesekera ROB (Ed.). The Medicinal Plant Industry. CRC Press, Boca Raton, FL.

Hikino H, Oshima Y, Suzuki Y, Konno C. 1985. Isolation and hypoglycemic activity of panaxans F, G, and H, glycans of *Panax ginseng* roots. Shoyakugaku Zasshi 39(4):331-333.

Hooper SN, Chandler RF. 1984. Herbal remedies of the Maritime Indians: phytosterols and triterpenes of 67 plants. Journal of Ethnopharmacology 10:181-194.

Hussey JS. 1974. Some useful plants of early New England. Economic Botany 28:311-337.

Isaacs HL. 1977. Iroquois herbalism: the past 100 years. International Journal of Social Psychiatry 22:272-281.

Iwabuchi H, Yoshikura M, Ikawa Y, Kamisako W. 1987. Studies on the sesquiterpenoids of *Panax ginseng* CA Meyer. Isolation and structure determination of sesquiterpene alcohols, panasinsanols A and B. Chemical Pharmaceutical Bulletin 35(5):1975-1981.

Jhang JJ, Staba EJ, Kim JY. 1974. American and Korean ginseng tissue cultures: growth, chemical analysis, and plantlet production. In Vitro 9:253-259.

Jie YH, Cammisuli S, Baggiolini M. 1984. Immunomodulatory effects of Panax ginseng CA Meyer in the mouse. Agents and Actions 15(3/4):386-391.

Jones NP. 1999. Quantitative ethnobotany of eastern Canada's First Nations Peoples and antifungal activity of traditional medicinal plants. Masters of Science dissertation, University of Ottawa, Ottawa, ON.

Kenarova B, Neychev H, Hadjiivanova C, Petkov VD. 1990. Immunomodulating activity of ginsenoside R_{g1} from *Panax ginseng*. Japan Journal of Pharmacology 54:447-454.

Khansari DN, Murgo AJ, Faith RE. 1990. Effects of stress on the immune system. Immunology Today 11(5):170-175.

Kim KH, Lee YS, Jung IS, Park SY, Chung HY, Lee IR, Yun YS. 1998. Acidic polysaccharide from *Panax ginseng* induces Th1 cell and macrophage cytokines and generates LAK cells in synergy with rIL-2. Planta Medica 64:110-115.

Kim SK, Sakamoto I, Morimoto K, Sakata M, Yamasaki K, Tanaka O. 1981. Seasonal variation of saponins, sucurose and monosaccharides in cultivated ginseng roots. Planta Medica 42:181-186.

Konno C, Murakami M, Oshima Y, Hikino H. 1985. Isolation and hypoglycemic activity of panaxans Q, R, S, T, and U, glycans of *Panax ginseng* roots. Journal of Ethnopharmacology 14(1):69-74.

Konoshima T. 1996. Anti-tumor-promoting activities of triterpenoid glycosides : cancer chemoprevention by saponins. In: Waller GR and Yamasaki K (Eds.), Saponins Used in Traditional and Modern Medicine. Plenum Press, New York, NY.

Kwon BM, Ro SH, Kim MK, Nam JY, Jung HJ, Lee IR, Kim YK, Bok SH. 1997. Polyacetylene analogs, isolated from hairy roots of *Panax ginseng*, inhibit acyl-CoA: cholesterol acyltransferase. Planta Medica 63:552-553.

Lee TM, Der Marderosian AH. 1981. Two-dimensional TLC analysis of ginsenosides from root of Dwarf ginseng (*Panax trifolius* L.) Araliaceae. Journal of Pharmaceutical Science 70(1):89-91.

Lee TM, Der Marderosian AH. 1988a. A chemical study of the saponins and flavonoids of Dwarf ginseng (*Panax trifolius* L.) and its comparison to related species in the Araliaceae. Proceedings of the 5th International Ginseng Symposium, Seoul, 1988.

Lee TM, Der Marderosian AH. 1988b. Studies on the constituents of Dwarf ginseng. Phytotherapy Research 2(4):165-169.

Lee YJ, Chung E, Lee KY, Lee YH, Huh B, Lee EK. 1997. Ginsenoside-R_{g1}, on the major active molecules from *Panax ginseng*, is a functional ligand of glucocorticoid receptor. Molecular and Cellular Endocrinology 133:135-140.

Lemaire I. 1991. Selective differences in macrophage populations and monokine production in resolving pulmonary granuloma and fibrosis. American Journal of Pathology 138: 487-495.

Lewis R, Wake G, Court G, Court JA, Pickering AT, Kim YC, Perry EK. 1999. Non-ginsenoside nicotinic activity in ginseng species. Phytotherapy Research 13:59-64.

Lewis WH, Zenger VE. 1982. Population dynamics of the American ginseng Panax quinquefolium (Araliaceae). American Journal of Botany 69(9):1483-1490.

Lewis WH, Zenger VE. 1983. Breeding systems and fecundity in the American ginseng, Panax quinquefolium (Araliaceae). American Journal of Botany 70(3):466-468.

Li HI. 1952. Floristic relationships between eastern Asia and eastern North America. Transactions of American Philosophical Society 42:371-429.

Li HI. 1972. Eastern Asia-eastern North America species-pairs in wide-ranging genera. In: Graham A. (Ed.), Floristics and paleofloristics of Asia and eastern North America. Elsevier, Amsterdam.

Li J, Huang M, Teoh H, Man RY. 1999a. *Panax quinquefolium* saponins protects low density lipoprotein oxidation. Life Science 64(1):53-62.

Li TSC, Mazza G, Cottrell AC, Gao L. 1996. Ginsenosides in roots and leaves of American ginseng. Journal of Agriculture Food and Chemistry 44:717-720.

Li Z, Guo YY, Wu CF, Li X, Wang JH. 1999. Protective effects of pseudoginsenoside-F_{11} on scopolamine-induced memory impairment in mice and rats. Journal of Pharmacy & Pharmacology 51(4):435-440.

Lien EJ, Gao H. 1990. Higher plant polysaccharides and their pharmacological activities. International Journal of Oriental Medicine 15(3):123-140.

Lim BO, Yu BP, Oh JH, Park DK. 1998. The inhibitory effects of ginsenoside and quercetin on oxidative damage by puromycin aminonucleoside in rat. Phytotherapy Research 12:375-377.

Linneau C. 1753. Species Planatarum 1058.

Linneau C. 1754. Genera Planatarum Ed 5. 481.

Linsefors L, Björk L, Mosbach K. Influence of elicitors and mevalonic acid on the biosynthesis of ginsenosides in tissue cultures of *Panax ginseng*. Biochemie und Physiologie der Pflanzen: 184: 413-418.

Liu CX, Xiao PG. 1992. Recent advances on ginseng research in China. Journal of Ethnopharmacology 36:27-38.

Liu J, Wang S, Liu H, Yang L, Nan G. 1995. Stimulatory effect of saponin from *Panax ginseng* on immune function of lymphocytes in the elderly. Mechanisms of Ageing and Development 83:43-53.

Luettig B, Steinmüller C, Gifford GE, Wagner H, Lohmann-Matthes ML. 1989. Macrophage activation by polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. Journal of the National Cancer Institute 81(9):669-675.

Lui JHC, Staba EJ. 1980. The ginsenosides of various ginseng plants and selected products. Journal of Natural Products 43(3):340-346.

MacCassady J. 1965. Some aspects of the chemistry of natural products. I. Screening of some native Ohio plants for alkaloids. Constituents of *Aralia racemosa*. Compounds related to pineal components. Dissertation abstract B26(5-6):2465-2466.

Maffei Facino RM, Carini M, Aldeni G, Berti F, Rossoni G. 1999. *Panax ginseng* administration in the rat prevents myocardial ischemia-reperfusion damage induced by hyperbaric oxygen: evidence for an antioxidant intervention. Planta Medica 65: 614-619.

Mathur A, Shukla YN, Pal M, Ahuja PS, Uniyal GC. 1994. Saponin production in callus and cell suspension cultures of *Panax quinquefolium*. Phytochemistry 35(5):1221-1225.

Mathur A, Mathur AK, Pal M, Uniyal GC. 1999. Comparison of qualitative and quantitative *in vitro* ginsenoside production in callus cultures of three *Panax* species. Planta Medica 65:484-486.

Matsunaga H, Katano M, Yamamoto H, Fujito H, Mori M, Takata K. 1990. Cytotoxic activity of polyacetylene compounds in *Panax ginseng* C.A. Meyer. Chemical and Pharmaceutical Bulletin 38(12):3480-3482.

Matsunaga H, Katano M, Saita T, Yamamoto H, Mori M. 1994. Potentiation of cytotoxicity of mitomycin C by a polyacetylenic alcohol, panaxytriol. Cancer Chemotherapy and Pharmacology 33:291-297.

Mihashi S. 1969. Further study on the diterpenes of *Aralia* spp. Tetrahedron Letters (21):1683-1686.

Mizuno M, Yamada J, Terai H, Kozukue N, Lee YS, Tsuchida H. 1994. Differences in immunomodulating effects between wild and cultured *Panax ginseng*. Biochemical and Biophysical Research Communications 200(3):1672-1678.

Moerman DE. 1998. Native American Ethnobotany. Timber Press, Portland, OR.

Nah SY. 1997. Ginseng; recent advances and trends. Korean Journal of Ginseng Science 21(1):1-12.

Nault A, White DJ. 1999. Update COSEWIC status report on American ginseng, *Panax quinquefolius*. Committee on the Status of Endangered Wildlife in Canada, Ottawa, Canada.

Oshima Y, Sato K, Hikino H. 1987. Isolation and hypoglycemic activity of Quinquefolans A, B, and C, glycans of *Panax quinquefolium* roots. Journal of Natural Products 50(2):188-190.

Peterson RL, Varney GT, Binns S. 1998. Structure of *Panax quinquefolius* L. (Araliaceae) roots with emphasis on secretory duct formation. Journal of the American Society for Horticultural Science 123(5):798-801.

Pritts KD. 1995. Ginseng: How to find, grow, and use America's forest gold. Stackpole Books, Mechanicsburg, PA.

Proctor JTA, Bailey WG. 1987. Ginseng: industry, botany, and culture. Horticultural Review 9:187-236.

Proctor JTA, Wang TS, Bailey WG. 1988. East meets west: cultivation of American ginseng in China. HortScience 23(6):968-972.

Quattrocchi U (Ed.). 2000. CRC World Dictionary of Plant Names. CRC Press, Boca Raton, FL.

Raman A, Houghton P. 1995. Ginseng. The Pharmaceutical Journal 254:150-152.

Raven PH, Evert RF, Eichhorn SE. 1986. Biology of Plants. Fourth Ed. Worth Publishers Inc., New York, NY.

Rimar S, Lee-Mengel M, Gillis CN. 1996. Pulmonary protective and vasodilator effects of a standardized *Panax ginseng* preparation following artificial gastric digestion. Pulmonary Pharmacology 9: 205-209.

Robbins CS. 1998. American Ginseng: The Root of North America's Medicinal Herb Trade. TRAFFIC North America, Washington, DC.

Salim KN, McEwen BS, Chao HM. 1997. Ginsenoside R_{b1} regulates ChAT, NGF, and trkA mRNA expression in the rat brain. Molecular Brain Research 47(1-2):177-182.

SAS Institute Inc. 1997. SAS version 6.12. SAS Institute Inc., Cary, NC.

Scaglione F, Cattaneo G, Alessandria M, Cogo R. 1996. Efficacy and safety of the standardized ginseng extract G 115 for potentiating vaccination against common cold and/or influenza syndrome. Drugs Experiment and Clinical Research 22(2):65-72.

Scaglione F, Cogo R, Cocuzza C, Arcidiacono M, Beretta A. 1994. Immunomodulatory effects of *Panax ginseng* C.A. Meyer (G115) on alveolar

macrophages from patients suffering with chronic bronchitis. International Journal of Immunotherapy 10(1):21-24.

Scaglione F, Ferrara F, Dugnani S, Falchi M, Santoro G, Fraschini F. 1990. Immunomodulatory activity of two extracts of *Panax ginseng* C.A. Meyer. Drugs Experiment and Clinical Research 16(10):537-542.

Schlessman MA. 1985. Floral biology of American ginseng (Panax quinquefolium). Bulletin of the Torrey Botanical Club 112 (2):129-133.

Schluter C, Punja ZK. 2000. Floral biology and seed production in cultivated North American ginseng (*Panax quinquefolius*). Journal of the American Society for Horticultural Science 125(5):567-575.

See DM, Broumand N, Sahl L, Tilles JG. 1997. In vitro effects of echinacea and ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. Immunopharmacology 35:229-235.

Shaw PC, But PPH. 1995. Authentication of *Panax* species and their adulterants by random-primed polymerase chain reaction. Planta Medica 61(5):466-469.

Shibata S, Mihashi S, Tanaka O. 1967. The occurrence of (-)pimarane-type diterpene in Aralia cordata Thunb. Tetrahedron Letters (51):5241-5243.

Shibata S, Tanaka O, Shoji J, Saito H. 1985. Chemistry and Pharmacology of *Panax*. In: Wagner H, Hikino H, Farnsworth NR. (Eds.). Economic and Plant Research Volume 1. Academic Press Inc., New York, NY.

Shin KS, Kiyohara H, Matsumoto T, Yamada H. 1998. Rhamnogalacturonan II dimers cross-linked by borate diesters from the leaves of *Panax ginseng* C.A. Meyer are responsible for expression of their IL-6 production enhancing activities. Carbohydrate Research 307:97-106.

Singh VK, George CX, Singh N, Agarwal SS, Gupta BM. 1983. Combined treatment of mice with Panax ginseng extract and interferon inducer. Planta medica 47: 234-236.

Small E. 1997. Culinary Herbs. National Research Council of Canada Monograph Publishing Program, NRC Research Press, Ottawa, ON.

Small E, Catling PM. 1999. Canadian Medicinal Crops. National Research Council of Canada Monograph Publishing Program, NRC Research Press, Ottawa, ON.

Smallfield BM, Follett JM, Douglas MH, Douglas JA, Parmenter GA. 1995. Production of Panax spp. in New Zealand. Acta Horticulturae 390:83-89.

Smith RG, Caswell D, Carriere A, Zielke B. 1996. Variation in the ginsenoside content of American ginseng, *Panax quinquefolius* L., roots. Canadian Journal of Botany 74(10):1616-1620.

Soldati F, Sticher O. 1980. HPLC separation and quantitative determination of ginsenosides from Panax ginseng, Panax quinquefolium and from ginseng drug preparations. Planta medica 38:348-357.

Soldati F, Tanaka O. 1984. *Panax ginseng*: relation between age of plant and content of ginsenosides. Planta Medica 50:351-352.

Song ZJ, Johansen HK, Faber V, Høiby N. 1997. Ginseng treatment enhances bacterial clearance and decreases lung pathology in athymic rats with chronic *P. aeruginosa* pneumonia. Acta pathologica, microbiologica et immunologica Scandinavica 105: 438-444.

Sonnenborn U, Proppert Y. 1991. Ginseng (*Panax ginseng* C.A. Meyer). British Journal of Phytotherapy 2(1):3-14.

Sonoda Y, Kasahara T, Mukaida N, Shimizu N, Tomoda M, Takeda T. 1998. Stimulation of interleukin-8 production by acidic polysaccharides from the root of *Panax ginseng*. Immunopharmacology 38:287-294.

Souto JT, Figueiredo F, Furlanetto A, Pfeffer K, Rossi MA, Silva JS. 2000. Interferon-gamma and tumor necrosis factor-alpha determine resistance to paracoccidioides brasiliensis infection in mice. American Journal of Pathology 156: 1811-1820.

Srividya S, Roy RP, Basu SK, Mukhopadhyay A. 2000. Scavenger receptor-mediated delivery of neuramyl depeptide activates antitumor efficacy of macrophages by enhanced secretion of tumor-suppressive cytokines. Journal of Leukocyte Biology 67: 683-690.

Statistics Canada. 2001. Background: ginseng production in Canada. http://res2.agr.ca/london/pmrc/english/study/newcrops/ginseng.html

Suzuki Y, Hikino H. 1989a. Mechanisms of hypoglycemic activity of panaxans A and B, glycans of *Panax ginseng* roots: effects on the key enzymes of glucose metabolism in the liver of mice. Phytotherapy Research 3(1):15-19.

SPSS for Windows. 1998. SYSTAT 9.01. SPSS Science Inc., Chicago, IL.

Tanaka O. 1987. Solubilizing properties of ginseng saponins. Korean Journal of Ginseng Science 11(2):145-153.

Tanaka O. 1990. Recent studies on glycosides from plant drugs of Himalaya and south-western China: chemogeographical correlation of *Panax* species. Pure and Applied Chemistry 62(7):1281-1284.

Tanaka O, Kasai R. 1984. Saponins of ginseng and related plants. In: Herz W, Grisebach H, Kirby GW, Tamm C (ed.). Progress in the Chemistry of Organic Natural Products 46 Springer-Verleg, Wien, NY.

Thompson GA. 1987. Botanical characteristics of ginseng. In: Craker LE, Simon JE (ed.). Herbs, Spices and Medicinal Plants: Recent Advances in Botany, Horticulture and Pharmacology vol. 2. Oryx Press, Phoenix, AZ.

Tomoda M, Hirabayashi K, Shimizu N, Gonda R, Ōhara N. 1994. The core structure of ginsenan PA, a phagocytosis-activating polysaccharide from the root of *Panax ginseng*. Biological Pharmaceutical Bulletin 17(9):1287-1291.

Tomoda M, Hirabayashi K, Shimizu N, Gonda R, Ōhara N. Takada K. 1993. Characterization of two novel polysaccharides having immunological activities from the root of *Panax ginseng*. Biological Pharmaceutical Bulletin 16(1):1087-1090.

Tomoda M, Takeda K, Shimizu N, Gonda R, Ōhara N, Takeda K, Hirabayashi K. 1993. Characterization of two acidic polysaccharides having immunological activities from the root of *Panax ginseng*. Biological Pharmaceutical Bulletin 16(1):22-25.

Trévoux A. 1717. Memoires pour l'histoire des Sciences et des beaux Arts. Reprinted in Slatkine Reprints, Genève.

Trévoux A. 1718. Memoires pour l'histoire des Sciences et des beaux Arts. Reprinted in Slatkine Reprints, Genève.

van Breeman RB, Huang CR, Lu ZZ, Rimando A, Fong HHS, Fitzloff JF. 1995. Electrospray liquid chromatography/mass spectrometry of ginsenosides. Analytical Chemistry 67(21):3985-3989.

Vuksan V, Sievenpiper JL, Wong J, Xu Z, Beljan-Zdravkovic U, Arnason JT, Assinewe V, Stavro MP, Jenkins AL, Leiter LA, 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.

Wagner H, Nörr H, Winterhoff H. 1994. Plant adaptogens. Phytomedicine 1:63-76.

Wakabayashi C, Murakami K, Hasegawa H, Murata J, Saiki I. 1998. An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. Biochemical and Biophysical Research Communications 246(3):725-730.

Washida D, Shimomura K, Nakajima Y, Takido M, Kitanaka S. 1998. Ginsenosides in hairy roots of a Panax hybrid. Phytochemistry 49(8):2331-2335.

Wen J, Zimmer EA. 1996. Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. Molecular Phylogenetics and Evolution 6(2):167-177.

White DJ. 1988. Status report on the American ginseng *Panax quinquefolium* in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa, ON.

Wielgorskaya T. 1995. Dictionary of Generic Names of Seed Plants. Columbia University Press, New York, NY.

Wróbel-Stermińska W, Tomczyk H. 1975. *Aralia racemosa* L.—Morphological and phytochemical investigations. Herba Polonica 83:247-252.

Xiao PG, Liu CX. 1999. Immunostimulants in traditional Chinese medicine. In: Wagner H (Ed.). Immunomodulatory Agents from Plants. Verlag Basel, Switzerland.

Yoshikawa M, Murakami T, Yashiro K, Yamahara J, Matsuda H, Saijoh R, Tanaka O. 1998. Bioactive saponins and glycosides. XI. Structures of new dammarane-type triterpenes oligoglycosides, Quinquenosides I, II, III, IV, and V, from American ginseng, the roots of *Panax quinquefolium* L. Chemical Pharmaceutical Bulletin 46(4):647-654.

Yuan CS, Attele AS, Wu JA, Liu D. 1998. Modulation of American ginseng on brainstem GABAergic effects in rats. Journal of Ethnopharmacology 62:215-222.

Zhong JJ, Bai Y, Wang SJ. 1996. Effects of plant growth regulators on cell growth and ginsenoside saponin production by suspension cultures of *Panax quinquefolium*. Journal of Biotechnology 45:227-234.

Zhong JJ, Wang DJ. 1996. Improvement of cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*: Cu^{2+} effect. Journal of Biotechnology 46:69-72.

Zhou J, Huang WG, Wu MZ, Yang CR, Feng KM, Wu ZY. 1975. Triterpenoids from Panax Linn. and their relationship with taxonomy and geographical distribution. Acta Phytotaxonomica Sinica 13(2):29-45.

APPENDIX

Wong SK, Asafu-Adjaye EB. April 2000. Interlaboratory collaborative study final report: Determination of ginsenosides (ginseng saponins) in *Panax* species and commercial products by HPLC method. AOAC Official Methods Program.

INTERLABORATORY COLLABORATIVE STUDY

FINAL REPORT

APRIL 2000

Collaborative Study Report

Determination of Ginsenosides (ginseng saponins) in *Panax* Species and Commercial Products by HPLC Method : Collaborative Study

Siu Kay Wong
Hong Kong Government Laboratory, 88 Chung Hau Street, Homantin Government Offices, Hong Kong.

Ebenezer B. Asafu-Adjaye
U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Product Quality Research Laboratory, Rockville, MD 20857.

Collaborators:
S.Y. Kwan, H.H.S. Fong, J. Fitzloff, H.W.Leung, J. Betz, H. Nicolidakis, K.C. Wen, X.R. Wang, J.T. Wang, G.A. Luo, J.X. Chen, G.Q.Li, J. Arnason, R.C. Lin and C.S. Kim.

ABSTRACT

Fifteen collaborating laboratories assayed four samples namely *Panax ginseng*, *Panax quinquefolius* and two ginseng products, for six ginsenosides. Collaborators also received a negative control sample for the recovery study. Reference standards were provided for the HPLC analysis and the system suitability test. The HPLC analyses were performed on the methanol extract of samples using UV detection at 203 nm. For *Panax* species, repeatability standard derivations (RSD_r) ranged from 3.63 to 6.60%; reproducibility standard derivations (RSD_R) ranged from 6.23 to 12.6%. For ginseng commercial products, repeatability standard derivations (RSD_r) ranged from 3.39 to 8.12%; reproducibility standard derivations (RSD_R) ranged from 6.47 to 13.7%. Recovery study was also conducted for three ginsenosides, i.e. R_{g1}, R_e and R_{b1}. The average recoveries were 99.9, 96.2 and 92.3 % respectively. However, based on the collaborators' results and feedback, it should be noted that the present method does not seem to be rugged enough for the determination of R_{g1} and R_e in ginseng product at levels below 300 mg/kg.

INTRODUCTION

More than 30 ginsenosides have been identified in different species and sub-species of the *Panax* plants [1]. The most abundant and common ginseng saponins are R_{g1}, R_e, R_{b1}, R_c, R_{b2} and R_d. They were reported to account for more than 90% of the total ginsenosides content of the root [2]. Several methods of identification and characterization have been reported [1,3-7], the most popular ones being HPLC [1,3-5,7]. Before HPLC, the most common method of identification has been thin-layer chromatography (TLC) [6,8,9], which is inherently inadequate for quantitative purposes. For some of these reported methods, peak resolution, particularly between Re and Rg1, was inadequate. Recently, more sophisticated and sensitive detection systems such as HPLC-evaporative light scattering detection (ELSD) [1], HPLC-MS [10,11] and HPLC-MS-MS [12] have been applied with very promising results. However, these methods are not economically feasible for routine operations.

In the light of the increasing use, and economic importance of ginseng products, the need to establish a simple yet accurate, reproducible, rugged and reliable method of quantitation of these ginsenosides becomes very important. Such a method may be used to standardize and control the manufacture process, and to establish the stability and purity of these products. This collaborative study is expected to produce a validated method that may be used for such purposes.

COLLABORATIVE STUDY

HPLC methods have been the most successful and now the most widely used analytical procedure for the determination of ginsenosides in *Panax* Species and related commercial products. A HPLC method proposed by the Product Quality Research Laboratory of the USFDA was submitted the AOAC Methods Committee for Nutrition and to fifteen laboratories for consideration to participate in a collaborative study. Upon approval of the study protocol, the participating laboratories were sent four samples and a negative control sample for the method validation study. The laboratories were instructed to keep the samples, store the reference standards and to perform the analysis within a month.

Samples for the collaborative study were prepared as follows:

Panax ginseng and *Panax quinquefolius*: Samples were provided by Prof. Harry Fong of the School of Pharmacy, University of Illinois, USA. Each of the two samples were all ground and mixed thoroughly as received. About 120 gram of each sample was taken and evenly divided into 20 portions. They were stored in plastic tube and screw-capped. To conduct the homogeneity test, four portions were randomly selected and the amount of ginsenosides were determined using

the same analytical procedures as in the study. Also, the presence of ginsenosides was confirmed by the LC/MS/MS technique. The samples were kept at room temperature inside a desiccator until they were delivered to the participants.

Commercial products:- Two different brands of ginseng tea powder were purchased from a local herb shop. They were then named as "Ginseng Product A" and "Ginseng Product B" respectively. For each of them, the content was thoroughly mixed and evenly divided into 20 portions and sealed. To conduct the homogeneity test, four portions were randomly selected and the amount of ginsenosides were determined using the same analytical procedures as in the study. Also, the presence of each ginsenosides was confirmed by the LC/MS/MS technique. The samples were kept at room temperature inside a desiccator until they were delivered to the participants.

Negative control sample:- *Panax ginseng* root sample with all ginsenosides removed by means of exhaustive solvent extraction. The efficiency of the removal of ginsenosides was confirmed using the same HPLC assay as used in this collaborative study.

Ginsenosides in *Panax* Species and related commercial products - HPLC Method
(Applicable to the determination of the ginsenosides from extracts of ginseng roots and commercial products at the mg/kg level)

Method performance

A. Principle

Ginsenosides were extracted with methanol from sample matrices. The sample extract was then concentrated and reconstituted with methanol. The ginsenosides were separated and determined by HPLC with UV detection at 203 nm. In order to separate the six ginsenosides of interest in a well-resolved manner within a single injection, gradient elution was employed.

B. Apparatus

(a) HPLC system - capable of running a solvent gradient has to be used. An autosampler for over-night unattended analysis is preferable.

(b) Detector - detection is by UV with the wavelength set at 203 nm.

(c) Column - a C_{18} reversed-phase, 5μm, 250 X 4.6 mm, connected with a C_{18} guard column.

(d) Injector - preferably be an automated one to minimize variability in the injection. The injection volume is 10 μL.

(e) Data system - integrator or computerized system such as Waters' Millennium® or HP ChemStation® is used.

C. Reagents

All reagents, including the ginsenoside standards, are analytical grade or higher. HPLC grade solvents are preferred as they can be used as received without further purification.

D. Preparation of the Apparatus

Before the analysis, the pump-lines have to be primed and the detector has to be stabilized.

E. Standard solutions

(1) *Calibration stock standard solution* - accurately weigh 24 mg each of the ginsenosides R_{b1}, R_{b2}, R_c, R_d, R_e, and R_{g1} in to a container. Add 16 mL of methanol and mix well to dissolve. To each of the labeled 5-mL volumetric flasks to be distributed to the participants, pipette 1 mL of the standard solution into it and evaporate the solvent under streams of nitrogen gas. Cap and seal the flasks with paraffin film. Deliver the flasks to the participants and instruct them to make up to the volume of the flasks with methanol. The final concentrations of the ginsenosides should then be 0.3 mg/mL. (2) *Quality control stock solution* - accurately weigh 48, 80 and 160 mg of ginsenosides R_{g1}, R_e and R_{b1} respectively in to a container. Add 16 mL of methanol and mix well to dissolve. To each of the labeled 10-mL volumetric flasks to be distributed to the participants, pipette 1 mL of the standard solution into it and evaporate the solvent under streams of nitrogen gas. Cap and seal the flasks with paraffin film. Deliver the flasks to the participants and instruct them to make up to the volume of the flasks with methanol. The final concentrations of the ginsenosides R_{g1}, R_e and R_{b1} should then be 0.3, 0.5 and 1.0 mg/mL respectively. (3) *Ginsenoside standard solutions used to establish retention times of different ginsenosides in the HPLC chromatogram* – weigh about 1.6 mg of ginsenoside R_{b1}. Add 16 mL of methanol and mix well to dissolve. To each of the labeled 2-mL micro-vials to be distributed to the participants, pipette 1 mL of the standard solution into it and cap the vials. Deliver the vials to the participants. Repeat the preparation for other ginsenosides R_{b2}, R_c, R_d, R_e, R_f and R_{g1} (4). *Working calibration standards* – prepare the working calibration standards solutions containing the six ginsenosides in concentrations of 0, 25, 50, 100, 200 and 300 μg/mL as shown in table below. Use volumetric or grade A graduated pipettes for the preparation.

Std. No.	Conc. Level, (μg/mL)	Preparation
Std. 6	300	Calibration stock solution
Std. 5	200	dilute 2 mL calibration stock solution to 3 mL
Std. 4	100	dilute 1 mL calibration stock solution to 3 mL
Std. 3	50	dilute 0.5 mL Std. 5 to 2 mL
Std. 2	25	dilute 0.5 mL Std. 4 to 2 mL
Std. 1	0	Use 3 mL methanol diluent

For each working calibration solution at different concentration levels, i.e. Std. 1 - Std. 6, dispense about 500 μL into four separate micro-vials and store in a freezer until required for analysis. On each day of analysis, one set of these calibration standard solutions is retrieved, brought to room temperature and used for analysis. **(5)** *Working Quality control standards* - Prepare the working QC solution, containing R_{bl}, R_e and R_{gl} at concentration 250, 125 and 75 μg/mL respectively, by mixing 0.5 mL quality control stock solution with 2 mL methanol. Use volumetric or "Grade A" graduated pipettes in the preparation. Prepare a set of eight working quality control standard solutions as above. Use one for the system suitability test. Use six for the intra-day validation test. For the one remaining working quality control standard, dispense about 500μL aliquots into three separate micro-vials and keep at –4°C until required for analysis. These will be used for the inter-day validation test. On three separate days of analysis, one of these is thawed and injected.

E. Mobile Phase

The mobile phase for the chromatography consists of *Solvent A:100% acetonitrile,* and *Solvent B: water*. The linear gradient program used is given below.

Time(min)	%A	%B	Flow rate, mL/min
0.0	20	80	1.6
20.0	20	80	1.6
60.0	42	58	1.6

G. Preparation of Samples

Accurately weigh a portion of sample (about 0.5 g for *Panax ginseng* and *Panax quinquefolius*, and about 1.0 g for ginseng products A and B) in a tared 50-mL Erlenmeyer flask. Add 15 mL of methanol and shake to mix. Sonicate the mixture at 25-30 °C for 30 min. Cool and filter through filter paper Whatman 40 or equivalent, into a 250-mL round-bottom flask, and return the residue to the Erlenmeyer flask. Add another 15 mL of methanol and sonicate the mixture at 25-30 °C for 30 min. Filter the extract into the same round-bottom flask, and wash the residue with methanol (3 x 15 mL) while on the filter. Evaporate the combined methanol extracts and washing to dryness, under *vacuum*, at 45-50°C. Redissolve the residue with methanol (4 x 2 mL), transfer the solution to a 10-mL volumetric flask, and make up to the volume with methanol. Filter the sample solution directly into the HPLC sample vial, using a filter cartridge, just prior to HPLC analysis. For each sample, conduct triplicate analysis concomitantly in the same day. Conduct a reagent blank analysis with the same procedure without adding any sample.

H. Preparation of Samples for the Recovery Study

Accurately weigh about 0.5 g of the negative control sample in a tared 50-mL centrifuge tube. Spike it with1.0 mL of quality control stock solution and mix thoroughly. Repeat the extraction procedure as described in Section G. Conduct three trials for the recovery study in the same day. Perform one blank extraction using the same procedure with negative control sample without the addition of standards.

I. HPLC Determination

(1) Identification of the Ginsenosides in the Chromatogram

Inject the standard solutions of the seven sealed micro-vials to establish the retention times of the seven ginsenosides for peak identification purposes. This is possible because previous [11] and recently published work [13] have shown that each eluted peak is due to just one ginsenoside, and that there were no co-eluting peaks. It should be noted that only six ginsenosides were quantitatively evaluated in this study.

(2) System Suitability

Perform the system suitability tests with one working quality control standard. After instrument equilibration, make a series of 10 injections (10 μL) using the instrumental set-up described above. From the chromatograms, calculate and report the capacity factor (k'), selectivity (α), no. of theoretical plates (N), resolution (R) between R_e and R_{g1}, and USP tailing factor (t). Also, calculate the precision or repeatability (RSD,%) for each of these parameters for the ginsenoside.

It is expected that the following values should be achieved in order to obtain good resolution for the peaks closely eluting peaks for R_{g1} and R_e.

Parameter	Value
Resolution, R	≥ 1.5
Selectivity, α	≅ 1.0
No. of theoretical plate, N	> 10,000
Capacity factor, k'	≅ retention time
USP Tailing factor, t	≅ 1.0

(3) Sample Analyses

Samples run on each day must be accompanied by one set of working calibration solutions (Std.1 - Std.6). Inject a working quality control standard to check the accuracy of calibration. Inject one set of working calibration solution in a way to bracket the sample extracts and working quality control standard injections. Construct a calibration curve with data obtained from the working calibration solutions for quantitation purpose.

(4) Intra-day Validation

Carry out the intra-day validation using the six working quality control standard together with a set of working calibration solutions on the same day. The six injections are made at equal intervals of time within the same day.

(5) Inter-day Validation

Carry out the Inter-day validation on three separate days of analysis by injecting, in triplicate, one of the working quality control working solution.

J. Calculation

For data plotted as (x-axis) = concentration of ginsenosides and (y-axis) = peak areas,

$$\text{Ginsenoside, mg/kg} = (\text{peak area}_{\text{sample}} - C) / S * D/W$$

where C = intercept of calibration curve, S = slope of calibration curve, D = dilution volume (mL) and W = sample weight (g)

If data is plotted as (x-axis) = peak areas and (y-axis) = concentration of ginsenosides,

$$\text{Ginsenoside, mg/kg} = [(\text{peak area}_{\text{sample}} \times S) + C] * D/W$$

where C = intercept of calibration curve, S = slope of calibration curve, D = dilution volume (mL) and W = sample weight (g)

RESULTS AND DISCUSSION

Fifteen laboratories from 6 countries and regions agreed to participate in this collaborative study and received the analytical protocol, samples and reference materials. However, only twelve laboratories reported their data before the deadline for the submission of results. The other three laboratories did not complete the study because of lack of time or facilities that were required.

Initial Data Screen

In the initial data screen, seven laboratories (2,6,7,10,11,12 and 13) were found to have some of their data deviating greatly from the respective average mean. The laboratories were contacted and requested to determine the causes and correct the erroneous results after explanation of error source. As reported in their replies, some of the erroneous results were due to calculation mistakes whereas others were due to errors in the matching of ginsenoside peaks in the chromatograms. However, some of the erroneous data could not be corrected, as the laboratories concerned could not identify the cause.

In the result sheets, laboratory 2 reported that the mobile phase system they used was different from that stipulated in the protocol. In response to our enquiry, the responsible laboratory remarked that problem of air bubbles formation in the LC mixing chamber was encountered when pure solvents (solvent A : acetonitrile and solvent B: water) were used to run the gradient. Therefore, they premixed the solvents to change the solvent A to 20% acetonitrile : 80 % water and solvent B to 40 % acetonitrile : 60 % water. The gradient was also changed as follows. 100 % solvent A: 0% solvent B was maintained for the first 20 min., then the ratio changed in a linear gradient to 0% solvent A : 100% solvent B in 40 min. As such, the acetonitrile/water ratio in the mobile phase throughout the LC analysis had deviated from what had been stipulated in the protocol. As only results from collaborators who had strictly followed the methodology under study should be included in order to truly reflect the method performance, data from Laboratory 2 were removed from the statistical analysis.

System Suitability Test Data

In the study, the collaborators were asked to perform system suitability test on certain

chromatographic parameters including capacity factor (k'), selectivity (α), no. of theoretical plates (N), resolution (R) and USP tailing factor (t). It was noted that the values obtained for individual parameter were consistent among the collaborators. Also, the values reported for individual parameter were within acceptable ranges as far as the purpose of the study was concerned. Particularly, R between R_{g1} and R_e was = 1.5.

Inter- and Intra-day Validation

The results indicated that eight collaborators could achieve a low %RSD value (i.e. well below 5%) for both inter- and intra-day reproducibility, whereas the %RSD values were within 10 % for the other collaborators except that inter-day reproducibility values of 13.9 and 23.9 % were reported by Laboratory 12 and 11 respectively. Further, it was observed that both Laboratory 11 and 12 had a relatively higher occurrence of outliers. It seems that there is a correlation ship between inter-day reproducibility and the occurrence of outlier results.

Statistical Analysis of Data

In the study, each participating laboratory analyzed two *Panax* species and two ginseng product samples for the presence of ginsenosides R_{g1}, R_e, R_{b1}, R_c, R_{b2} and R_d, and submitted the results listed in Tables 1-4. Laboratory 2's data were not included in the statistical analysis; they are given only for comparison purposes.

The procedures and criteria outlined in the AOAC manual [14] for determining outliers in the data were followed. Also, the spreadsheet program provided along with the manual was employed for the statistical analysis of the data, and the results are summarized in Tables 5-8. Overall, not more than two outliers were found in the determination of individual ginsenoside in the four test samples. However, more than five outliers were found in the determination of R_{g1} and R_e in the ginseng product B. Three collaborators had forwarded some possible reasons for the discrepancy encountered. Laboratory 1 remarked that low concentration of the ginsenosides R_{g1} and R_e in the sample as well as the presence of interference peaks in the chromatograms would be the cause of the problem. Laboratory 5 added that the problem might be due to poor resolution and integration of the R_{g1} and R_e peaks. In this respect, Laboratory 5 suggested that columns with much better resolving power be used. Also, Laboratory 13 considered that the mobile phase gradient used in the study might be a cause of the problem. In the study, the ginsenosides R_{g1} and R_e were eluted at times closed to the start of the gradient where due to the abrupt change in the mobile phase composition, disturbance might be experienced. Because of this, the resolution of the two ginsenosides might be affected and thus cause large variances in the peak integration. Based on the collaborators' results and feedback, it seems that the present

method may not be rugged enough for the analysis of R_{g1} and R_e in ginseng product at levels below 300 mg/kg. To truly reflect the method performance for cases not affected by such limitation, we suggested excluding them in the overall statistical analysis. As such, the results of the statistical analysis are as follows. For *Panax* species, repeatability standard derivations (RSD_r) ranged from 3.63 to 6.60%; reproducibility standard derivations (RSD_R) ranged from 6.23 to 12.6%. For ginseng commercial products, repeatability standard derivations (RSD_r) ranged from 3.39 to 8.12%; reproducibility standard derivations (RSD_R) ranged from 6.47 to 13.7%. The average recoveries were 99.9, 96.2 and 92.3 % for R_{g1}, R_e and R_{b1} respectively.

Collaborator's comments

The comments from the collaborators are summarized in Table 9. Regarding the comments from Laboratories 1 and 4 about the occurrence of precipitation during the reconstitution step, both had not reported any adverse effect on their results due to the phenomenon.

ACKNOWLEDGMENTS

We are grateful to the following collaborators and their associates for their cooperation in this study.

- Sik-yiu Kwan, Government Laboratory, Hong Kong SAR, P.R. China.
- Jing-tao Wang, The University of Nanjing, Department of Pharmacognosy, Nanjing, P.R.China.
- Guo-an Luo, The University of Tsinghua, Department of Chemistry, Beijing, P.R.China.
- Xiao-ru Wang, The University of Xiamen, Department of Chemistry, Xiamen, P.R. China.
- Harry Fong and John Fitzloff, The University of Illinois, College of Pharmacy, Chicago, Illinois, USA.
- George Qian Li, The University of Sidney, Department of Pharmacy, Sidney, Australia.
- John Arnason, The University of Ottawa, Department of Biology, Ottawa, Canada.
- Hin-wing Yeung and Hei-wun Leung, The Baptist University, Institute for the Advancement of Chinese Medicine, Hong Kong SAR, P.R. China.
- Jiang-xing Chen, The Shanghai Institute for the Drug Control, Shanghai, P.R.China.
- Rui-chao Lin, The National Institute for the Control of Pharmaceutical & Biological Products, Beijing, P.R. China,
- Joe Betz, The Centre for Food Safety & Applied Nutrition, USFDA, Division of Natural Products, Washington, DC, USA.
- Erick Suen and Kuo-ching Wen, National Laboratory of Food & Drugs, Taipei, Taiwan, Republic of China.

- Helen Nicolidakis and Kenneth Breakell, The Health Department, Health Protection Branch, Burnaby, Canada.
- Hoon Park and Choen-suk Kim, Korean Ginseng & Tobacco Research Institute, Taegeon, Korea.

REFERENCES

[1] M. K. Park, J. H. Park, S. B. Han, Y. G. Shin and I. H. Park, *J. Chromatogr. A,* **736** (1996) 77-81.

[2] G. Sollorz, *Deutsch. Apoth. Ztg.* **125** (1985) 2025.

[3] F. Soldati and O. Sticher, *J. Med. Plant Res.*, **38** (1980) 348-357.

[4] B. Meier, A. Meier-Bratschi, K. Dallenbach-T Π lke and O. Sticher, in H. M. Chang, H. W. Yeung, W. W. Tso and A. Koo, (Editors), *Advances in Chinese Medicinal Materials Research,*. World Scientific Publ. Co., Singapore (1985) 471-484.

[5] Wen-Fa Sye and Pai-Feng Tsai, *J. Chin. Chem. Soc.*, **34** (1987) 1-6.

[6] D. Xin, W. Wen, S. Yuqing and H. Yongli, *Chin. J. Chromatogr.* **12**(3), (1994) 173-174.

[7] Wu-Chang Chuang and Shuenn-Jyi Sheu, *J. Chromatogr. A,* **685** (1994) 243-251.

[8] J. Hon-Chiu Lui and E. John Staba, *J. Natural Products*, **43(3)** (1980) 341-347.

[9] G.H. Jolliffe and N.M.Din, *Pakistan J. Sci. Ind. Res.*, **26(2)** (1983) 76-78.

[10] Y. N. Elkin, V. V. Mahankov, L:. Uvarova, P. V. Bondarenko, R. A. Zubarev and A. N. Knysh, *Acta Pharmacologica Sinica,* **14** (2) (1993) 97-100.

[11] R.B. van Breemen, C.H. Huang, Z.E. Lu, A. Rimando, H.H.S. Fong, J.F. Fitzloff, *Anal. Chem.* **67** (1995) 3985-3989.

[12] X. Wang, T. Sakuma, E. B. Asafu-Adjaye and G. K. Shiu, *Pharm. Res.* **14**(11) (1997) S-591.

[13] X. Wang, T. Sakuma, E. B. Asafu-Adjaye and G. K. Shiu, *Anal. Chem.*, **71**,1579-1584.

[14] AOAC International (1993) AOAC Official Methods Program, Manual on Development, Study, Review, and Approval Process for AOAC Official Methods", AOAC, Arlington, VA.

Table 1. Collaborative data for the determination of ginsenosides in *Panax ginseng* [a]

Laboratory	R_{g1}			R_e			R_{b1}		
1	3170	3140	3200	3560	3570	3540	6780	6940	6680
2[b]	4190	4058	4543	2920	2942	2990	5174	5228	5929
4	2677	2678	2677	2957	2958	2877	5754	6195	6153
5	2880	3020	3140	3270	3440	3580	7510	7680	7700
6	3317	3061		3496	3388		6981	6739	
7	2699	2855	2802	3052	3158	3087	7842	9001	7892
9	3209	2913	2913	3580	3179	3431	7478	6693	7343
10	2796	2711	2769	3126	3051	3080	7524	7208	7455
11	2456	2956	3020	2602	3047	3125	5891	7004	6539
12	175[R]	167[R]	172[R]	173[R]	167[R]	175[R]	440[R]	424[R]	435[R]
13	2539[r]	3304[r]	4630[r]	4392[r]	2857[r]	3106[r]	499[R]	513[R]	521[R]
15	2953	3001	3060	3242	3247	3230	6075	6023	6236

Laboratory	R_c			R_{b2}			R_d		
1	4480	4370	4490	3470	3510	3450	2900	2880	2930
2[b]	3830	4327	4514	3284	3620	3663	2555	2587	2740
4	4236	4317	4355	3237	3317	3236	2637	2678	2797
5	5050	5140	5150	3780	3680	3710	3190	3320	3290
6	4362	4653	4794	3642	4038		3076	3028	
7	4896	4813		3339	3352	3660	3278	3658	3589
9	5335	4682	5294	4060	3537	4008	3351	2855	3283
10	4954	4718	4791	3847	3607	3601	2957	2943	3006
11	3260[R]	4003[R]	3725[R]	3081	3707	3512	2867	3514	3252
12	248 R	238[R]	245[R]	216[R]	218[R]	221[R]	198[R]	245[R]	251[R]
13	3866	4071	4113	2947	3081	3098	2544	2652	2718
15	4876	5075	5147	3522	3735	3783	3040	3175	3289

[a] units are mg/kg.

[b] data not included in the statistical analysis.

[r] repeatability outlier

[R] reproducibility outlier.

Table 2. Collaborative data for the determination of ginsenosides in *Panax quinquefolius* [a]

Laboratory	R_{g1}			R_e			R_{b1}		
1	1740	1760	1790	12300	12300	12300	30200	31100	30200
2[b]	1509	1837	1950	10319	10989	12277	29635	29681	32764
4	1599	1518	1519	11113	10982	10592	27224	27995	27498
5	1650	1670	1690	12200	12300	12400	31000	30900	31800
6	1721	1933	1649	12092	14164	12176	28038	34084	29636
7	1759	1701	2043	10244[r]	6787[r]	9006[r]	28302	28123	30313
9	1532	1586	1571	12605	12438	12437	29950	29068	32680
10	1604	1574	1620	12058	11841	12079	30881	30434	31137
11	1644	1664	1779	11710	10794	10606	33413	32928	31133
12	1932	1696	1725	12667	12473	12571	36981[R]	38160[R]	38160[R]
13	1747	2116	2162	13373	13528	15318	26880	27873	29531
15	1741	1673	1651	12597	12673	12813	30322	29588	31098

Laboratory	R_c			R_{b2}			R_d		
1	3210	3140	3130	434	436	443	6060	6240	6050
2[b]	2766	2829	3153	1199	1031	911	6122	5962	6137
4	2798	2835	2838	420	419	400	5597	5631	5795
5	3140	3240	3400	480	486	495	6400	6420	6500
6	3537	3592	3109	768	810	680	6034	7315	6879
7	3425	3397	3873	372	394	472	5059	4838	6020
9	3030	3144	3035	453	498	464	6071	6220	6094
10	3295	3126	3229	544	498	·618	6229	5947	6174
11	3081	3170	2955	521	497	493	6312	6491	6627
12	3423	3515	3586	1273[R]	1251[R]	1245[R]	7018	7517	7603
13	2725	2926	3213	452	486	511	5836	6145	6422
15	3388	3303	3323	397	381	387	6556	6296	6466

[a] units are mg/kg. [b] data not included in the statistical analysis.

[r] repeatability outlier. [R] reproducibility outlier.

Table 5. Collaborative data for the determination of ginsenosides in Ginseng Product A

Laboratory	R_{g1}			R_e			R_{b1}		
1	268	274	282	2210	2160	2190	7100	6990	6990
2[b]	306	345	341	2265	2067	2143	7138	6526	7128
4	270	270	260	1849	1899	1898	5957	6078	6005
5	207	237	284	2080	2420	2240	7890	7930	7560
6	215	233	203	1928	1923	1634	5951	5918	4986
7	233	200	255	1932	2075	2131	6946	6671	7734
9	245	268	255	2000	2170	2015	6031	6561	6293
10	N	N	N	1199[R]	1209[R]	1189[R]	6815	6909	6803
11	188	207	246	1914	1941	1703	6731	7282	7377
12	263	249	257	2088	2048	2152	7605	6325	6873
13	336[r]	460[r]	549[r]	1894	1804	1853	5917	5031	5245
15	280	280	274	2212	2308	2262	6663	6767	6732

Laboratory	R_c			R_{b2}			R_d		
1	1760	1620	1630	228	241	231	3020	2950	3000
2[b]	2029	1638	1766	531	429	514	3019	2835	3001
4	1509	1529	1519	240	250	250	2549	2609	2628
5	1970	1960	1870	348[R]	302[R]	291[R]	3460	3310	3170
6	1525	1517	1277	240	237	193	2608[R]	2590[R]	2184[R]
7	1634	1767	1986	254	225	269	2546	2825	2907
9	1581	1708	1591	245	265	248	2613	2824	2626
10	1666	1701	1701	280	252	244	2880	2945	2864
11	1798	1822	1714	252	255	236	3060	3142	2832
12	1666	1354	1493	166[r]	311[r]	365[r]	3108	2472	2650
13	1510	1295	1335	234	204	208	2575	2205	2283
15	1760	1813	1761	232	238	229	2869	2991	2906

[a] units are mg/kg

[b] data not included in the statistical analysis

[r] repeatability outlier

[R] reproducibility outlier

[N] no data submitted

Table 4. Collaborative data for the determination of ginsenosides in Ginseng Product B[a]

Laboratory	R_{g1} [#]			R_e [#]			R_{b1}		
1	199	196	190	299	292	320	810	836	812
2[b]	140	126	138	357	334	341	646	707	752
4	160	150	150	310	340	340	810	800	770
5	155	131	132	447	449	424	917	907	911
6	121	121		465	410		730	699	
7	332	311	298	208	213	185	1010[R]	906[R]	942[R]
9	249	205	212	301	309	339	840	794	805
10	N	N	N	N	N	N	834	935	876
11	87	139	57	350	251	102	905	858	728
12	99	90	57	343	465	406	514	467	425
13	269	251	147	355	370	375	104[R]	108[R]	109[R]
15	212	210	209	385	378	378	832	840	805

Laboratory	R_c			R_{b2}			R_d		
1	600	613	618	385	376	379	557	575	570
2[b]	711	793	826	477	454	456	729	642	703
4	620	670	870	430	400	400	550	550	580
5	652	651	665	401	400	405	558	549	573
6	569	557		335	322		475	469	
7	642	567	623	542[R]	517[R]	588[R]	736[R]	755[R]	821[R]
9	667	650	718	435	404	409	538	500	578
10	602	664	652	427	419	439	562	579	605
11	467[R]	472[R]	389[R]	448	433	423	623	789	650
12	235[R]	227[R]	222[R]	215[R]	209[R]	216[R]	588[r]	245[r]	376[r]
13	634	677	689	408	407	399	515	571	620
15	678	689	669	392	430	440	721	721	725

[a] units are mg/kg

[b] data not included in the statistical analysis

[r] repeatability outlier

[R] reproducibility outlier

[N] no data submitted

[#] the results were not included in the statistical analysis for reasons stated in the "Results and Discussion" Section

Table 5. Statistical data from the determination of ginsenosides in *Panax* ginseng

Statistic	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d
Mean, mg/kg	2940	3230	6970	4700	3550	3060
Repeatability, r	396	378	988	476	504	459
Repeatability standard deviation, S_r	142	135	353	170	180	164
Relative repeatability standard deviation, RSD_r, %	4.82	4.17	5.07	3.63	5.09	5.37
Reproducibility, R	596	725	2210	1160	834	840
Reproducibility standard deviation, S_R	213	259	788	414	298	300
Relative reproducibility standard deviation, RSD_R, %	7.27	8.01	11.3	8.81	8.42	9.80

Table 6. Statistical data from the determination of ginsenosides in *Panax quinquefolius*

Statistic	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d
Mean, mg/kg	1720	12300	30100	3220	490	6270
Repeatability, r	311	1540	4000	423	90.4	885
Repeatability standard deviation, S_r	111	549	1430	151	32.3	316
Relative repeatability standard deviation, RSD_r, %	6.46	4.46	4.76	4.71	6.60	5.05
Reproducibility, R	451	2830	5150	731	173	1680
Reproducibility standard deviation, S_R	161	1010	1840	261	61.7	599
Relative reproducibility standard deviation, RSD_R, %	9.38	8.18	6.12	8.13	12.6	9.56

Table 7. Statistical data from the determination of ginsenosides in ginseng product A

Statistic	R_{g1}	R_e	R_{b1}	R_c	R_{b2}	R_d
Mean, mg/kg	248	2030	6630	1650	240	2830
Repeatability, r	56.3	283	1020	277	43.1	442
Repeatability standard deviation, S_r	20.1	101	366	98.8	15.4	158
Relative repeatability standard deviation, RSD_r, %	8.12	4.98	5.53	6.00	6.40	5.60
Reproducibility, R	81.2	526	2190	530	53.8	815
Reproducibility standard deviation, S_R	29.0	188	782	189	19.2	291
Relative reproducibility standard deviation, RSD_R, %	11.7	9.25	11.8	11.5	7.99	10.3

Table 8. Statistical data from the determination of ginsenosides in ginseng product B

Statistic	R_{g1}[#]	R_e[#]	R_{b1}	R_c	R_{b2}	R_d
Mean, mg/kg			784	640	403	584
Repeatability, r			145	73.4	38.4	110
Repeatability standard deviation, S_r			51.7	26.2	13.7	39.4
Relative repeatability standard deviation, RSD_r, %			6.6	4.09	3.39	6.75
Reproducibility, R			342	116	86.2	224
Reproducibility standard deviation, S_R			122	41.4	30.8	80.0
Relative reproducibility standard deviation, RSD_R, %			15.6	6.47	7.65	13.7

[#] The results were not included in the statistical analysis for reasons stated in the "Results and Discussion" Section

List of Participating Laboratories

No.	Participating Laboratory*
1	Baptist University, Institute for the Advancement of Chinese Medicine, Hong Kong SAR, P.R. China.
2	Department of Health, National Laboratories of Foods and Drugs, Republic of China.
3	Government Laboratory, Hong Kong SAR, P.R.China.
4	Health Department, Health Protection Branch, Canada.
5	Korean Ginseng & Tobacco Research Institute, Taegeon, Korea.
6	National Institute for the Control of Pharmaceutical & Biological Control, Beijing, P.R. China.
7	Shanghai Institute for Drug Control, Shanghai, P.R. China.
8	U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Product Quality Research Laboratory, Rockville, USA.
9	U.S. Food and Drug Administration, Center for Food Safety & Applied Nutrition, Division of Natural Products, Washington, DC, USA.
10	University of Illinois, College of Pharmacy, Chicago, USA.
11	University of Nanjing, Department of Pharmacognosy, Nanjing, P.R.China.
12	University of Ottawa, Department of Biology, Ottawa, Canada.
13	University of Sidney, Department of Pharmacy, Sidney, Australia,
14	University of Tsinghua, Department of Chemistry, Beijing, P.R. China.
15	University of Xiamen, Chemistry Department, Xiamen, P.R. China.

Note : * The laboratories are listed in alphabetical order, and not according to the Laboratory Code used in the study.

Table 9. Comments from collaborators

Laboratory No.	Comment
1	a. Precipitates were observed when the residues of ginseng product A and B were re-dissolved with methanol. b. Low concentration of the ginsenosides R_{g1} and R_e in the ginseng product B as well as the presence of interference peaks in the chromatograms would be the cause of the discrepancy in the determination.
4	a. Precipitates were observed when the residues of ginseng product A and B were re-dissolved with methanol. b. Two ghost peaks at about 55 and 56 min. were observed in the next chromatogram everytime after an injection of ginseng product B extract.
5	a. The discrepancy in the determination of R_{g1} and R_e in ginseng product B might be due to poor resolution and integration of the R_{g1} and R_e peaks. Hence, columns with much better resolution capability should be used.
6	a. The extraction procedure should be simplified such that results with better repeatability might be achieved.
12	a. The sample taken for the analysis of ginseng product A & B should be reduced from 1.0 g to 0.5 g. As such, the peak shape of the analytes might be improved.
13	a. The problem encountered in the determination of R_{g1} and R_e in ginseng product B might be related to the mobile phase gradient used. In the study, the ginsenosides R_{g1} and R_e were eluted out at times closed to the start of the gradient of the mobile phase system, when disturbance might be experienced due to the abrupt change in the mobile phase composition. Because of this, the resolution of the two ginsenosides might be affected and hence caused large variances in the peak integration.

Appendix : Typical LC chromatograms

1. Chromatogram of the reagent blank
2. Chromatogram of the mixed ginsenosides standard
3. Chromatogram of the *Panax ginseng* sample extract
4. Chromatogram of the *Panax quinquefolius* sample extract
5. Chromatogram of the "Ginseng Product A" sample extract
6. Chromatogram of the "Ginseng Product B" sample extract

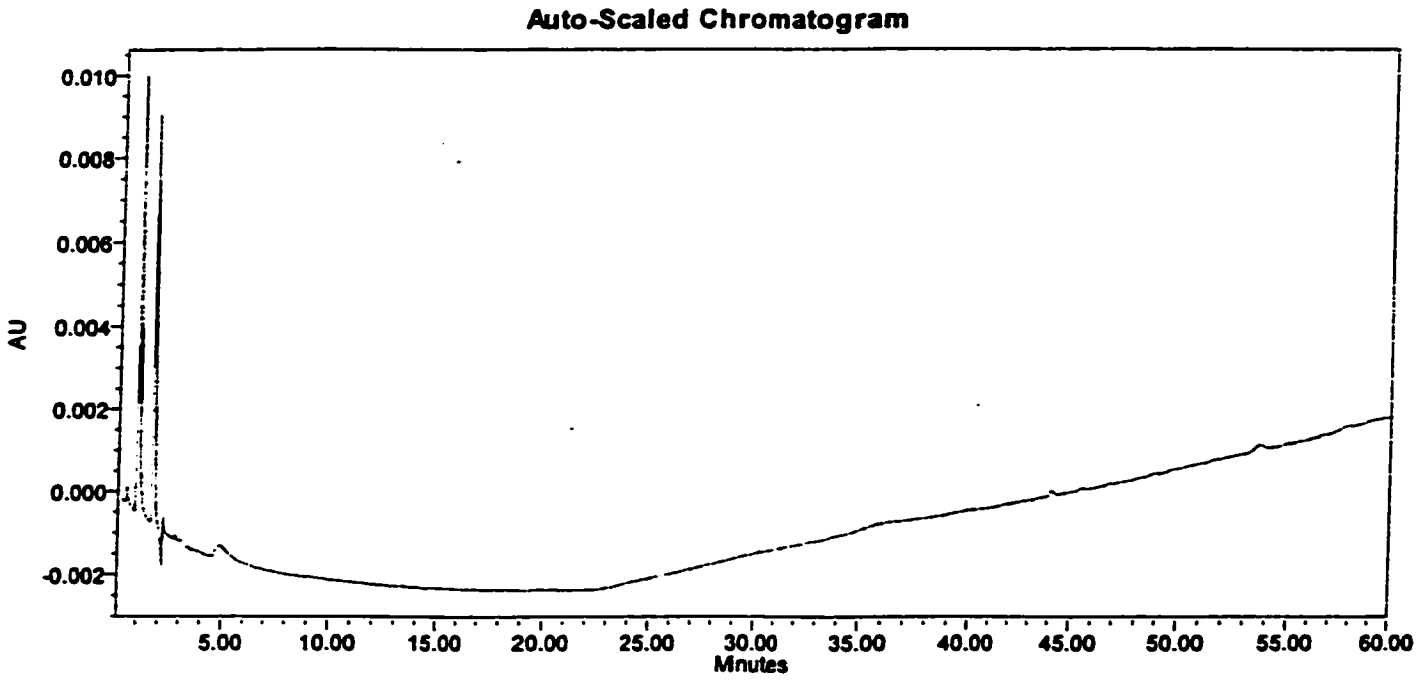


Figure 1 : HPLC Chromatogram of the reagent blank

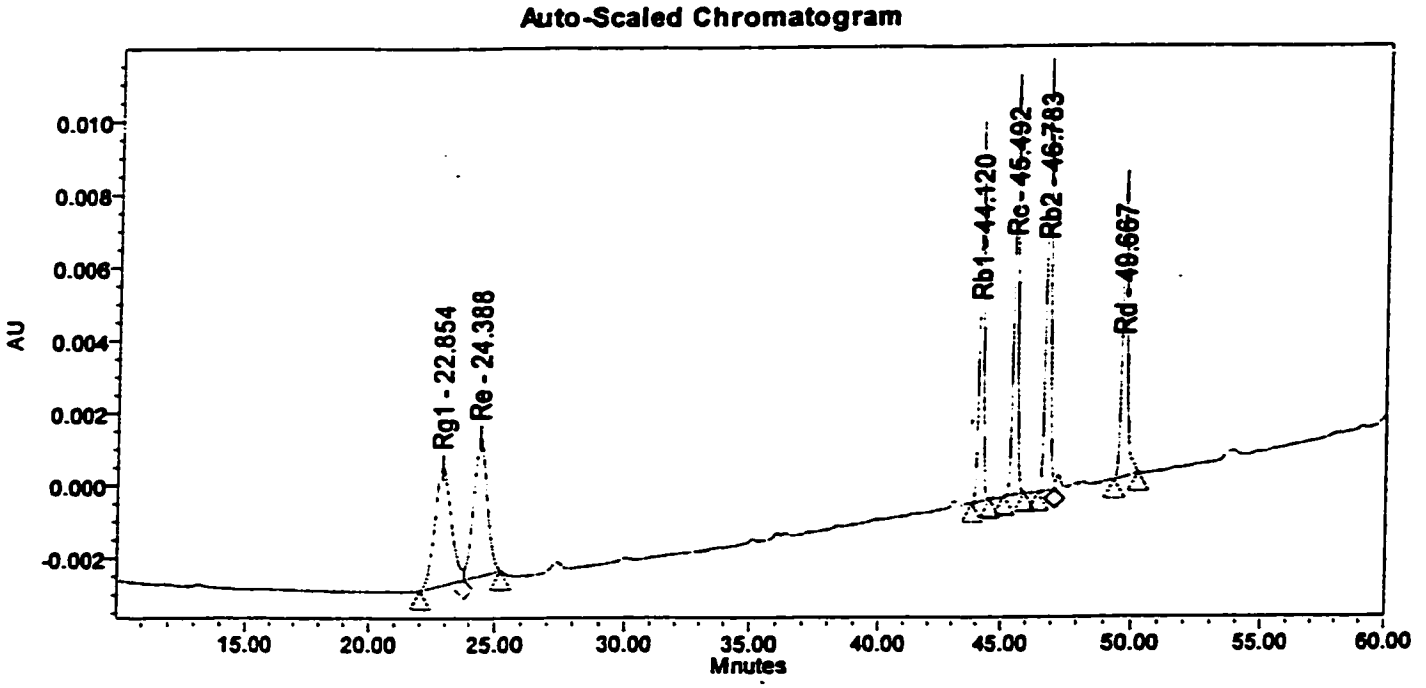


Figure 2 : HPLC Chromatogram of the mixed ginsenosides standard

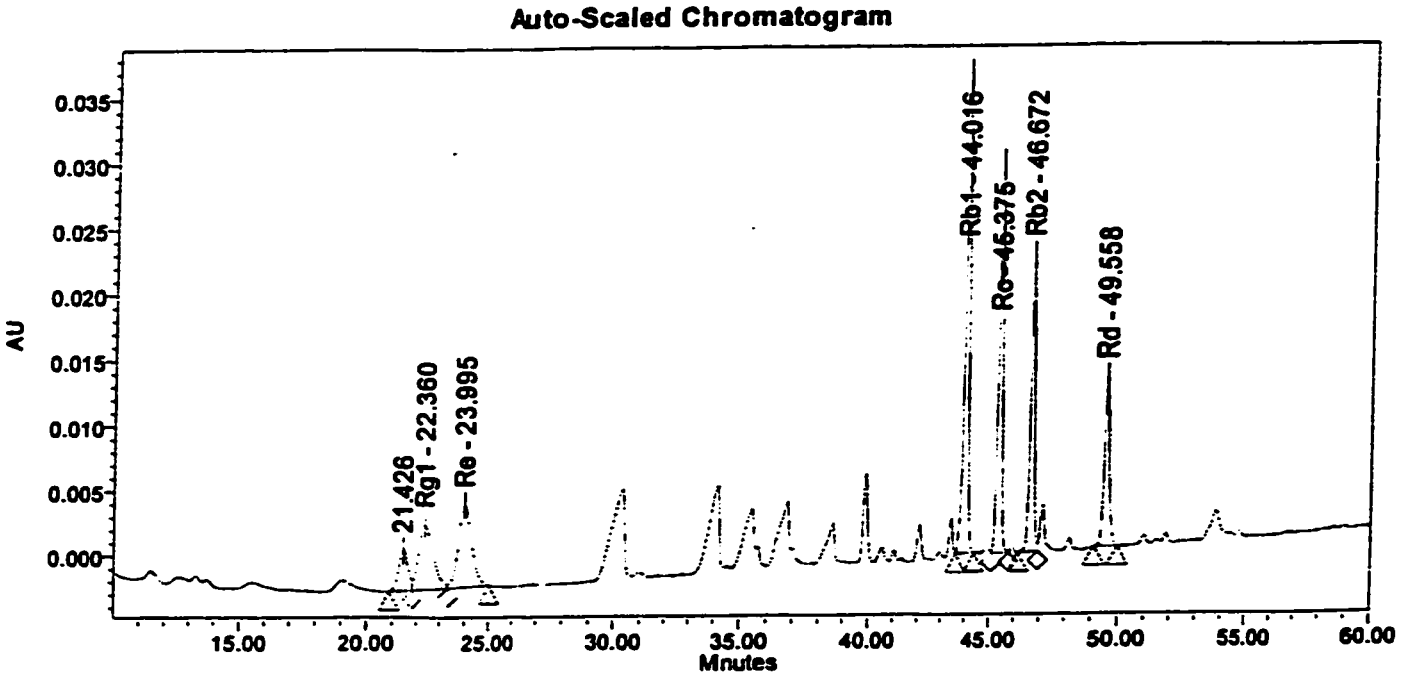


Figure 3 : HPLC Chromatogram of the *Panax ginseng* sample extract

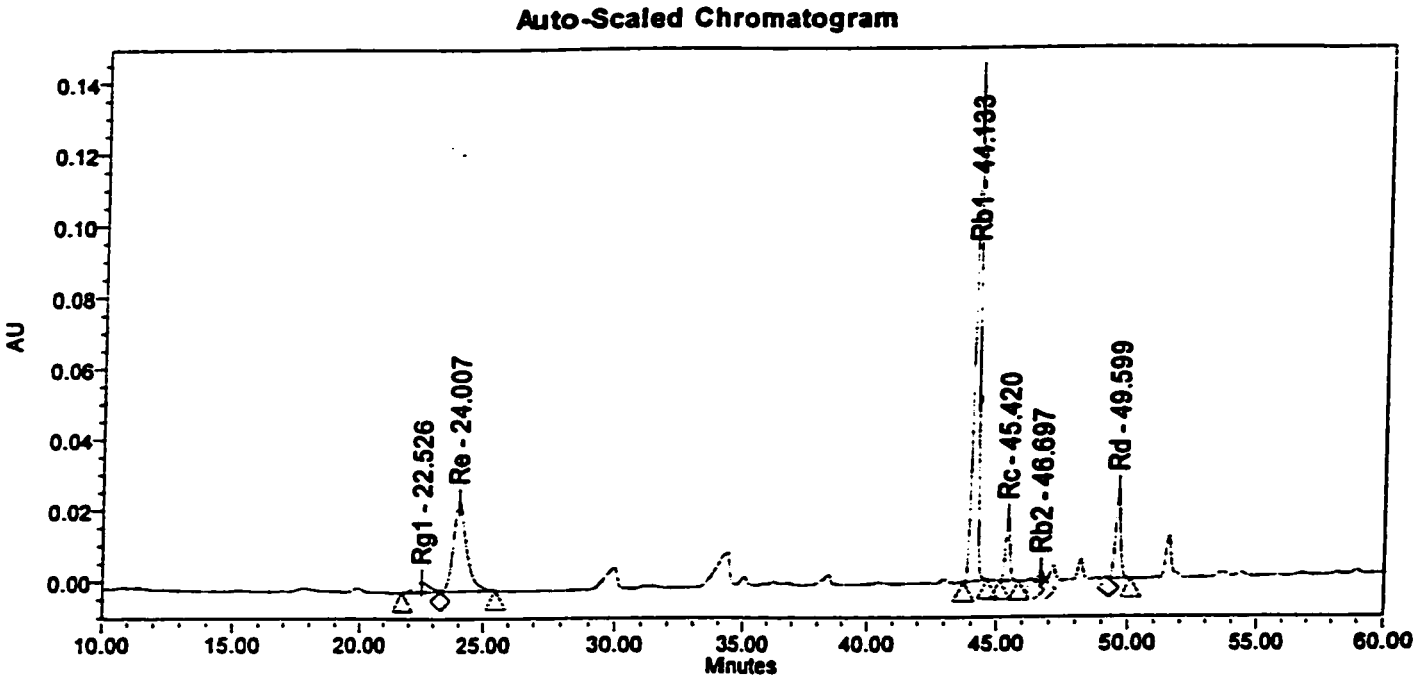


Figure 4 : HPLC Chromatogram of the *Panax quinquefolius* sample extract

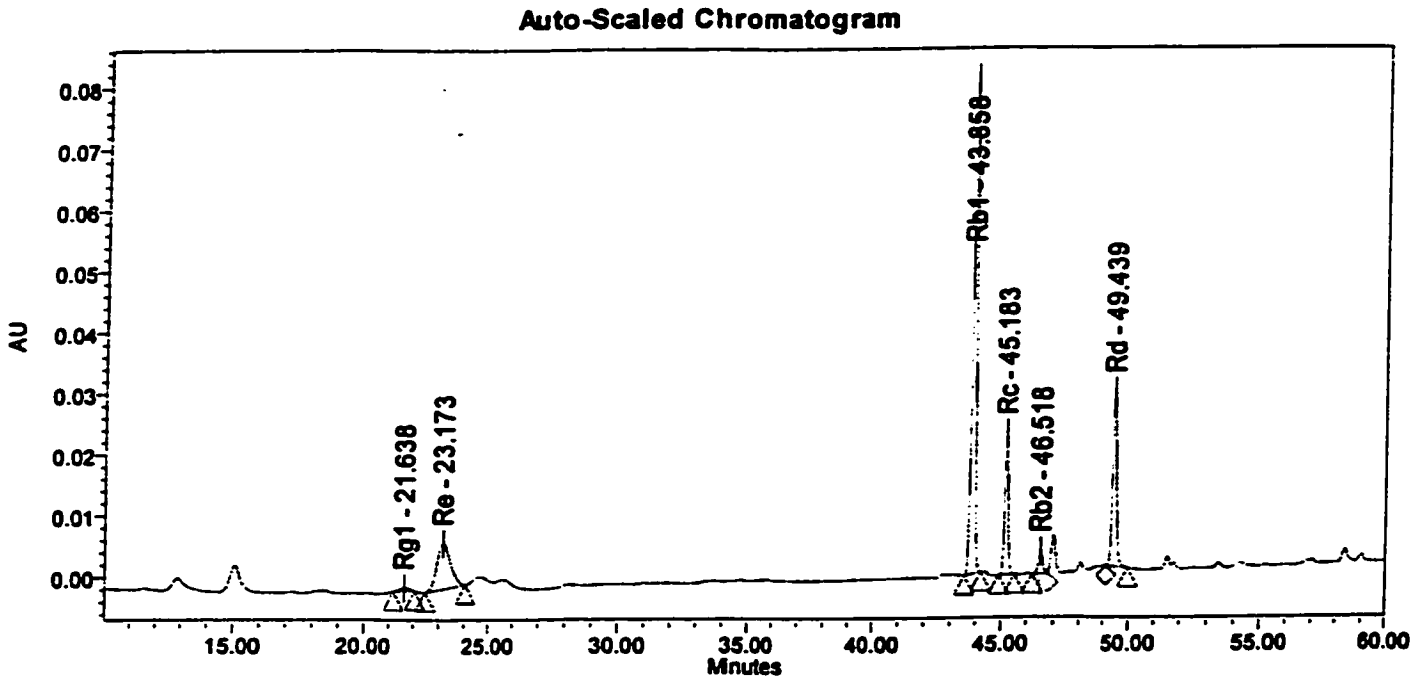


Figure 5 : HPLC Chromatogram of the "Ginseng Product A" sample extract

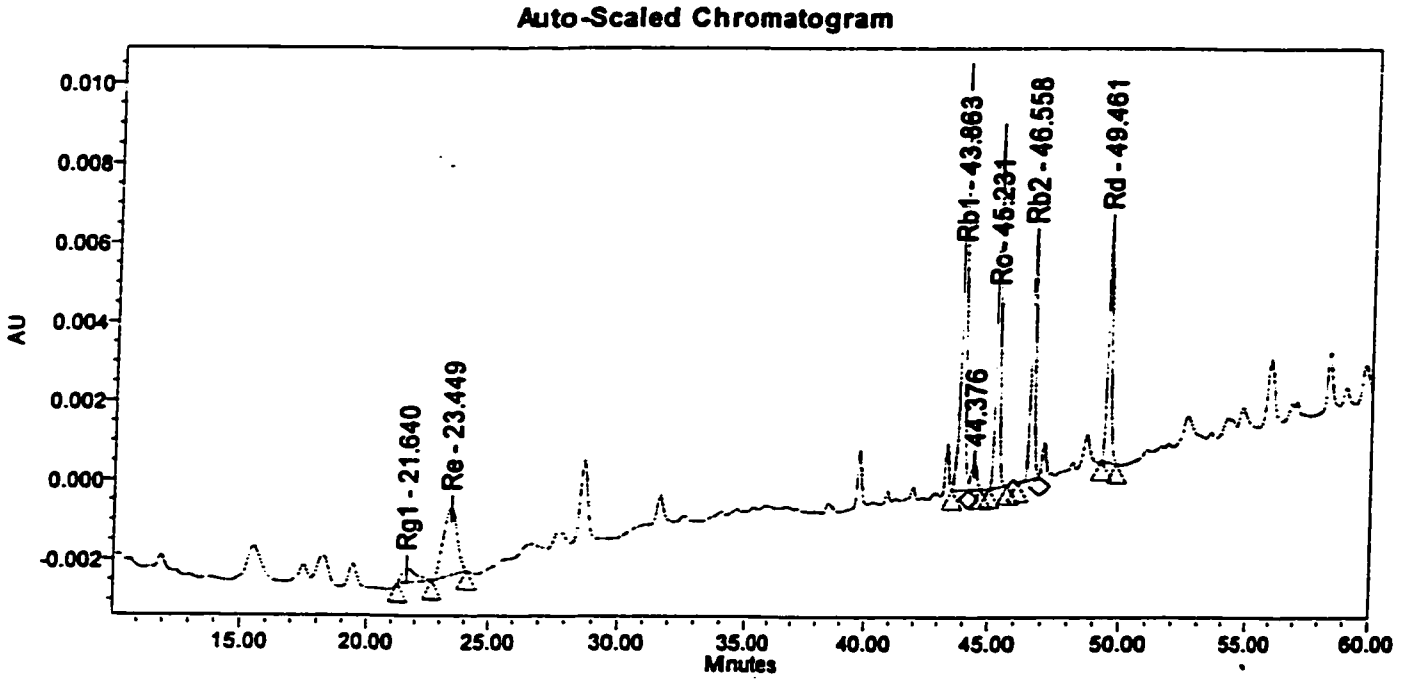


Figure 6 : HPLC Chromatogram of the "Ginseng Product B" sample extract