CHEMICAL STUDIES OF *PANAX NOTOGINSENG* AND RELATED SPECIES, AND EVALUATION OF POTENTIAL ANTIPLATELET AND ANTICOAGULANT EFFECTS

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LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications

1. **Lau AJ**, Koh HL. Quality control of herbs: principles and procedures, using *Panax* as an example. In: Leung PC, Fong H, Xue CCL, eds. *Annals of Traditional Chinese Medicine*, *Current review of Chinese medicine—quality control of herbs and herbal materials*, vol. 2. Singapore: World Scientific Publishing Co.; 2006: Chapter 6, 87-115.

2. Hong DY, **Lau AJ**, Yeo CL, Liu XK, Yang CR, Koh HL, Hong Y. Genetic diversity and variation of saponin contents in *Panax notoginseng* roots from a single farm. *J. Agric. Food Chem.* 2005; 53: 8460-8467.

3. Koh HL, **Lau AJ**, Chan ECY. Hydrophilic interaction liquid chromatography with tandem mass spectrometry for the determination of underivatized dencichine (β-*N*-oxalyl-L-α,β-diaminopropionic acid) in *Panax* medicinal plant species. *Rapid Commun. Mass Spectrom.* 2005; 19: 1237-1244.

4. **Lau AJ**, Seo BH, Woo SO, Koh HL. High-performance liquid chromatographic method with quantitative comparisons of whole chromatograms of raw and steamed *Panax notoginseng*. *J. Chromatogr. A* 2004; 1057: 141-149.

5. **Lau AJ**, Woo SO, Koh HL. Analysis of saponins in raw and steamed *Panax notoginseng* using high performance liquid chromatography with diode array detection. *J. Chromatogr. A* 2003; 1011: 77-87.

6. **Lau AJ**, Holmes MJ, Woo SO, Koh HL. Analysis of adulterants in a traditional herbal medicinal product using LC-MS-MS. *J. Pharm. Biomed. Anal.* 2003; 31: 401-406.

Conference presentations

1. **Lau AJ**, Yeo CL, Hong DYQ, Liu XK, Yang CR, Hong Y, Koh HL. A study on the saponin contents and genetic diversity in individual *Panax notoginseng* roots from a good agricultural practice farm. Poster presentation at: *18th Singapore Pharmacy Congress*; July 1-2, 2006; Singapore.

2. **Lau AJ**, Chan EC, Koh HL. Analysis of dencichine, a haemostatic agent, in *Panax* species using hydrophilic interaction chromatography-tandem mass spectrometry. Oral and poster presentations at: *Inaugural AAPS-NUS Student Chapter Symposium*; September 16, 2005; Singapore. (Best presenter award)

3. **Lau AJ**, Chan EC, Koh HL. Liquid chromatography-tandem mass spectrometry for the determination of dencichine, a haemostatic agent in *Panax* medicinal plant

species. Oral presentation at: *Inaugural Inter-varsity Symposium, 17th Singapore Pharmacy Congress*; July 1-3, 2005; Singapore. (Best presenter award)

4. **Lau AJ**, Tanaka N, Chan EC, Koh HL. Determination of dencichine in *Panax* species using liquid chromatography-tandem mass spectrometry. Poster presentation at: *Inaugural International Congress on Complementary and Alternative Medicines (ICCAM)*; February 26-28, 2005; Singapore.

5. **Lau AJ**, Seo BH, Woo SO, Koh HL. Chromatographic pattern matching of raw and steamed *Panax notoginseng*. Poster presentation at: *15th International Symposium on Pharmaceutical and Biomedical Analysis*; May 2-6, 2004; Florence, Italy.

6. **Lau AJ**, Seo BH, Woo SO, Koh HL. Chromatographic pattern matching of *Panax notoginseng*, a Chinese herbal medicine. Poster presentation at: *16th Singapore Pharmacy Congress*; November 22-23, 2003; Singapore. (Best poster award-1st prize)

7. **Lau AJ**, Woo SO, Koh HL. Analysis of raw and steamed *Panax notoginseng* using HPLC-DAD. Poster presentation at: *AAPS Annual Meeting and Exposition*; November 10-14, 2002; Toronto, Canada.

Provisional patent

1. Koh HL, Lau AJ. Anti-thrombotic activities of extracts and components from raw and steamed *Panax notoginseng*, US Provisional Patent, No. 60/828,078, 4th Oct 2006.

TABLE OF CONTENTS

SUMMARY

The overall objectives of this work are to develop methods for the quality control of *Panax notoginseng*, and to study the effects of processing on the chemical and biological differences between raw and steamed *P. notoginseng*.

A new HPLC-DAD method has been developed and validated for the analysis of saponins in raw and steamed *P. notoginseng* roots, and in products from various sources. *P. ginseng* and *P. quinquefolium* were also compared to *P. notoginseng*. Simultaneous quantification of six saponins (R1, Rg1, Re, Rb1, Rc and Rd) in *P. notoginseng* showed that the concentrations of these saponins decreased significantly upon steam processing. A chromatographic pattern matching analysis tool was employed, optimised and successfully applied to the differentiation of the roots and products, showing that it is a useful tool in assessing the quality of herbal products.

Key marker compounds in the extract of steamed *P. notoginseng* which differentiate the two forms were isolated and identified. Their identities were 20S-ginsenoside Rh1, 20R-ginsenoside Rh1, 20S-ginsenoside Rg3, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5. This is the first report of isolation of ginsenosides Rk1 and Rk3 from *P. notoginseng* roots.

Besides saponins, *P. notoginseng* is known to contain dencichine, a bioactive polar amino acid derivative with haemostatic activities. In this work, a novel HILIC/ESI-MS/MS method was successfully developed and validated for the analysis of underivatised dencichine in *Panax* species, providing rapid analysis in five minutes, high selectivity and sensitivity without the need for sample derivatisation. Raw *P. notoginseng* samples were found to have significantly higher quantities of dencichine than steamed samples, and the concentrations of dencichine in *P. ginseng* and *P.*

quinquefolium were significantly lower than those in raw *P. notoginseng*, thereby explaining their different indications.

Haematological activities (platelet aggregation and blood coagulation activities) of the raw and steamed samples and their key chemical components were investigated *in vitro* and *ex vivo*. Steamed samples resulted in significantly greater inhibition of platelet aggregations and longer coagulation times than raw samples. *P. ginseng* and *P. quinquefolium* generally exhibited lower activities. Ginsenoside Rg5 (98 μM) and 20S-ginsenoside Rg3 (92 μM) have better antiplatelet activities compared to aspirin (131 μM), indicating that they are potential leads for antiplatelet drugs. *In vivo* tail bleeding time (haemostatic) assays further showed that both forms of *P. notoginseng* have anti-haemostatic activities, with the steamed form being significantly more effective than the raw form.

In conclusion, the results support the hypothesis that steaming of raw *P. notoginseng* roots changes the concentration and composition of chemical components in *P. notoginseng*. The two forms and its related species have been successfully differentiated. In addition, the chemical changes upon steam processing have an important impact on their activities, with the steamed form and some of its components having potentially good antithrombotic activities. The methods developed in this work can be further optimised for the quality control of other botanical medicine.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

β-ODAP	β-*N*-oxalyl-L-α,β-diaminopropionic acid
α-ODAP	α-*N*-oxalyl-L-α,β-diaminopropionic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ANN	Artificial neural networks
AP-PCR	Arbitrarily-primed polymerase chain reaction
APTT	Activated partial thromboplastin time
Ara(f)	Arabinose in furanose form
Ara(p)	Arabinose in pyranose form
AT-III	Antithrombin III
ATP	Adenosine triphosphate
CAM	Complementary and alternative medicine
cAMP	cyclic adenosine monophosphate
CE	Capillary electrophoresis
CMC	Carboxymethylcellulose
CNS	Central nervous system
CPM	Chinese Proprietary Medicine
DAD	Diode array detector
DAP	Diaminopropionic acid
DNA	Deoxyribonucleic acid
ED_{50}	Effective dose for 50% of population
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector
ESI	Electrospray ionisation
FDA	Food and Drug Administration
FDP	Fibrinogen degradation products
FT-IR	Fourier-transformed infra-red
GAP	Good Agricultural Practice
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GCP	Good Clinical Practice
Glc	Glucose
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GSP	Good Supply Practice
HCA	Hierarchical clustering analysis
HILIC	Hydrophilic interaction chromatography
HMWK	High molecular weight killikrein
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IC_{50}	50% inhibitory concentration
i.e.	That is
IR	Infra-red
KNN	K-nearest neighbours
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry

LD_{50}	Lethal dose for 50% of population
LDA	Linear discriminant analysis
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MEKC	Micellar electrokinetic chromatography
MRM	Multiple reaction monitoring
NC	No coagulation
NCCAM	National Center for Complementary and Alternative Medicine
NIR	Near infrared
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
OPT	o-phthalaldehyde
OVB	Owren's Veronal Buffer
PBS	Phosphate buffered saline
PCA	Principal component analysis
PMSD	Pattern match standard deviation
PNS	*P. notoginseng* saponins
ppm	Parts per million
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rha	Rhamnose
RSD	Relative standard deviation
S/N	Signal to noise ratio
SD	Standard deviation
SIMCA	Soft independent modelling of class analogy
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TT	Thrombin time
TxA_2	Thromboxane A_2
UV	Ultraviolet
vWF	von Willibrand factor
WHO	World Health Organisation
Xyl	Xylose

CHAPTER 1

INTRODUCTION

1.1 Herbal medicines

1.1.1 Importance of herbal medicines

Traditional medicine, as defined by the World Health Organisation (WHO), refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being [WHO, 2003a]. In industrialised countries, adaptations of traditional medicine are termed complementary and alternative medicine (CAM), and it is often used interchangeably with traditional medicine. CAM is also broadly defined by National Center for Complementary and Alternative Medicine (NCCAM) as a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine [NCCAM, 2006; Barnes *et al*., 2004]. Herbal medicines (also known as botanical medicines, phytomedicines, natural products), which is a part of traditional medicine or CAM, refers to any herbs, herbal materials, herbal preparations and finished herbal products [WHO, 2002].

According to World Health Organisation [WHO, 2003a], the global market for herbal medicines currently stands at over US$60 billion annually and this figure is growing steadily, with a projected US$400 billion market by 2010 [Wang and Ren, 2002]. It is estimated that 65-80% of the world's population use traditional medicine as the primary form of healthcare [WHO, 2003a]. Traditional herbal preparations account for 30-50% of the total medicinal consumption in China. In the United States, 158 million of the adult population use complementary medicines and according to

the USA Commission for Alternative and Complementary medicines, US$17 billion was spent on traditional remedies in 2000 [WHO, 2003a]. In one of the most comprehensive updated survey on CAM [Barnes *et al*., 2004], 62% of U.S. adults used some form of CAM and natural products is among the top three most prevalent types of CAM used by about 19% of the population. In the United Kingdom, annual expenditure on alternative medicines is US$230 million [WHO, 2003a]. These statistics showed the growing worldwide importance of herbal medicines in both developing and industrialised countries. In developing countries, the broad use of herbal medicines is often attributed to its accessibility, affordability and their cultural beliefs. While in many developed countries, the increasing use of herbal medicines is often fuelled by concerns regarding adverse effects and unsatisfactory treatments from modern western drugs, the need for apparently milder treatments for chronic debilitating diseases, and greater public access to health information [WHO, 2002].

In response to the widespread use of herbal medicines, there is growing awareness of its safety, efficacy, quality and regulatory control by healthcare professionals, regulatory authorities of different countries and the public. Policy makers are faced with these challenges and are developing various strategies for ensuring good practices of herbal medicines and its integration into modern medicine. Western medicine emphasises the use of a rigorous scientific approach. However, for herbal medicine, such scientific evidences are still far from sufficient to meet the criteria needed to support its worldwide use. Fortunately, research on herbal medicines has been increasing over the years and these can be seen by the increase in funding and establishment of CAM research and research units in both developed and developing countries [WHO, 2002; NCCAM, 2004].

1.1.2 Safety of herbal medicines

1.1.2.1 Incidences of adverse effects

Despite the common belief that natural herbal medicines are safer than western medicine, there are risks and adverse effects associated with herbal medicines. Due to inadequate documentations, at present, there is still limited data to indicate reliable incidence figures for adverse events related to herbal medicines. In an active adverse drug reaction reporting programme in a Taiwan hospital, Chinese crude drugs were responsible for 22% of hospital admissions and 12% of all adverse effects [Ernst, 2004]. In two general wards of a Hong Kong hospital, 0.2% of cases were due to adverse reactions to traditional Chinese herbal medicines [Ernst, 2004]. Australian practitioners of traditional Chinese medicine estimated an average of 1.4 adverse events during each year of full-time practice.

1.1.2.2 Intrinsic adverse effects

The adverse effects or toxicities resulting from herbal medicines can be classified into two main categories. The first category is the intrinsic or plant-associated health risks due to active ingredients in the plant. These can be predictable, dose-dependent reactions due to their pharmacological effects (Type A) or idiosyncratic reactions not predictable from their pharmacology (Type B), such as allergy and anaphylaxis. For herbal medicines, Type C adverse reactions involve those that are pharmacologically predictable and develop gradually during long-term use (e.g. slowed bowel function upon long term use of stimulant herbal laxatives), while Type D reactions are effects with a latency period of months or years (e.g. mutagenic effects) [Ernst, 2005a]. Type A dose-dependent reactions with herbal preparations will also include effects with deliberate overdose or accidental poisoning

and interactions with pharmaceuticals [Drew and Myers, 1997; Pinn, 2001, Myers and Cheras, 2004]. As herbal medicines are often taken complementarily with therapeutic drugs, drug-herb interactions have been a major safety concern, especially when healthcare professionals are often unaware of the type of herbal medicines that patients have been self-administering. These interactions may increase or decrease the pharmacological or toxicological effects of the drugs or herbs. Some of the well-known interactions with clinical significance have been extensively reviewed [Fugh-Berman, 2000; Hu *et al*., 2005; Tirona and Bailey, 2006]. Therefore, to ensure the safety of complementary medicines, the Australia New Zealand Therapeutic Products Authority [ANZTPA, 2006] has recently recommended a two-tier regulatory system based on the level of risk of the medicine.

1.1.2.3 Extrinsic adverse effects

The second category is extrinsic or non-plant-associated adverse effects, which include factors such as contamination (with heavy metals, pesticides, micro-organisms, microbial toxins, radioactive substances etc), adulteration (accidental or intentional), misidentification, substitution, lack of standardisation, incorrect preparation/ dosage, and inappropriate labelling/ advertising [Drew and Myers, 1997]. These additional extrinsic factors make it more complicated for health professionals to assess the adverse effects of herbal preparations, as compared to conventional pharmaceuticals. Heavy metals are sometimes added intentionally for traditional uses, or it can arise unintentionally from environmental, cultivation and manufacturing processes. The levels of contamination have to be controlled to prevent heavy metal toxicities. Adulteration with synthetic drugs is a problem which may result in serious adverse effects in patients. For example, the addition of steroids, tranquillisers, non-

steroidal anti-inflammatory drugs (NSAIDs) or phosphodiesterase-5 inhibitors, into Chinese herbal preparations increases the likelihood of effectiveness but may place patients at risk of their adverse effects, and over-dosage of the drugs (if they are already taking these prescribed drugs). In fact, several herbal products have caused toxicity and have been withdrawn from the market due to the presence of these synthetic drugs, and toxic heavy metals such as mercury, arsenic, lead and copper [Ernst, 2004; Koh and Woo, 2000]. This issue is further discussed in Section 1.1.4.3. Misidentification often occurs when the plant species look similar or when several similar but confusing names are used. This may result in erroneous usage, with potential clinical implications. Toxic reactions have been reported when the plants have been substituted with another similar but toxic plant. One classic example is *Stephania tetrandra* (Fen fangji), a traditional diuretic, anti-rheumatic and pain reliever, which have been mistakenly substituted with *Aristolochia fangchi* (Guang fangji). Both herbs have rather similar external morphology and Chinese names. The latter contain nephrotoxic components, the aristolochic acids, which result in many cases of serious renal failure [Pinn, 2001]. Furthermore, substitution with inferior commercial varieties by unethical practices may result in potential inefficacy and adverse effects. The different preparation or processing methods for herbal medicines can also increase the efficacy or reduce the toxicity of some herbs, so incorrect preparation methods may result in adverse effects. Similar to western medicines, correct labelling is also important to provide the right information and it should not mislead patients. In view of this problem, some countries (e.g. Singapore, Hong Kong) have regulations to control the label contents of herbal products. To add on to the complexity, the therapeutic and toxic components of plants may vary due to many environmental factors, cultivation and post-harvesting conditions, so these variations

in their components due to lack of standardisation may lead to inefficacy or potential adverse effects in some cases.

From the above intrinsic and extrinsic factors, it can be seen that naturally occurring herbs are not necessarily harmless despite being natural. Long traditional history of usage is not a guarantee for its safety and the risks of herbal medicines need to be evaluated systematically with safety and toxicological studies, as well as post marketing surveillance studies [WHO, 2004]. These problems of herbal medicines highlighted the importance of implementing good quality control, standardisation and improved regulations/ policies to ensure their safety.

1.1.3 Efficacy of herbal medicines

The efficacy of herbal medicines is often based on traditional uses and claims. Although several herbal medicines have a long history of use, this does not guarantee their efficacy. Healthcare professionals practice modern medicine, which is based on evidence-based medical science, so it is difficult for them to accept treatments that lacked sound scientific data to support its efficacy claims [Mahady *et al*., 2001]. Furthermore, practitioners may not be able to recommend and advise patients on herbal medicines accurately without well-established efficacy data. Therefore, there is a need for rigorous scientific investigations in order to prove and explain the uses, discover new uses, understand the mechanisms of actions and finally, to bring herbal medicines into mainstream medicine. Similar to western medicine, *in vitro* and *in vivo* studies are often used to first test its efficacy in the initial preclinical stages. Well-designed, multi-centred, randomised, double-blind, placebo-controlled clinical studies involving significant number of human subjects are then needed to prove the efficacy of herbal medicines in humans. Although anecdotal reports of utility are of interest,

particularly in giving indications of the herbal medicines worthy of intensive study, they may not be viewed as a substitute for detailed scientific studies and clinical trials. In recent years, there are increasing numbers of randomised clinical trials of herbal medicines being published and systematic reviews/ meta-analyses of these studies have become increasingly available [Ernst, 2005b].

However, the evaluation of pre-clinical and clinical efficacy of herbal medicines is a more challenging and complicated process than synthetic medicines [Fong *et al*., 2006]. Some traditional effects or terms used in traditional medicine (e.g. yin and yang) are difficult to prove using modern scientific methods. Furthermore, herbal medicines contain a range of pharmacologically active compounds and it is usually not known which compounds are important for therapeutic effects. Isolated components may have different effects from the whole plant extracts. According to traditional practice, efficacy is often attributed to multiple components in the extracts and this concept is increasingly being accepted by many countries and the WHO [Xie and Wong, 2005]. The different components may have synergistic, cumulative, complementary effects or even antagonistic effects. This is an area where there is much speculation but relatively little concrete knowledge to date. These multi-component characteristics of herbal medicines render efficacy testing more complex [Ernst, 2005b]. The current methods of standardisation of a few components may not be sufficient to ensure consistencies of the whole herbal extracts, as variation of other components may still remain. As a result of such lack of quality control, reproducibility of efficacy studies may be affected. Therefore, the key challenges to efficacy and safety assessments, as summarised by Fong *et al*. [2006], are the quality of raw materials, appropriateness of the activity assessment, data interpretation,

standardisation methodology, pharmacokinetics and bioavailability of the active components, dosage formulation, clinical study designs and outcome measures.

1.1.4 Quality of herbal medicines

Safety, efficacy and quality are three inter-related factors which are essential for all synthetic medicines. As both the safety and efficacy are mainly affected by the type and amount of chemical constituents and contaminations present, quality assurance of herbal medicines is critical and proper regulations are needed to ensure compliance to the quality standards.

1.1.4.1 Factors affecting quality

Unlike synthetic drugs, herbal medicines present a set of unique problems when quality aspects are considered. The variations of botanicals are due to several factors such as strains and species differences, organ specificity, climate, geography, seasonal variation, cultivation, harvesting, storage, transportation, post-harvest treatment, manufacturing practices, adulteration, substitution and contamination [Mahady *et al*., 2001].

Botanicals of the same species may have different genetic makeup or belong to different strains or varieties. For example, the accumulation of hypericin in *Hypericum perforatum* is greater in the narrow leafed populations than the broader leafed varieties. It is also known that closely related species in the same family differ in their contents. Therefore, it is necessary that the starting material is accurately identified by their Latin scientific names and authenticated. The use of common names is inadequate as they may refer to more than one species. The site of chemical biosynthesis, accumulation and storage are also different in different plant parts, e.g.,

the ginsenosides content in *Panax ginseng* leaves and roots are different, and hence it is important that the right plant part is used.

Seasonal variation is an important factor affecting chemical accumulation. Depending on the plant, accumulation of chemical constituents can occur at any time during the various stages of their growth. Different times of the same day may even influence the chemical contents for some plants such as *Ginkgo biloba* [Scholten, 2003]. Therefore, plants may need to be harvested during specific periods. The cultivation methods, climate and conditions such as soil, light, water, temperature, nutrients further complicate the phytochemical accumulation. The physical appearance and chemical quality of botanicals can also be influenced by methods employed in field collection, harvesting, post-harvesting and manufacturing methods, shipping and storage. Moreover, contaminations by micro-organisms, fungi, aflatoxins, chemical agents (pesticides, herbicides, heavy metals), inorganic matters, as well as by foreign organic matters such as other plant parts, insect parts, animal parts and excreta during any stages of plant material production can affect its quality and lead to unsafe products [Mahady *et al*., 2001]. In view of the numerous influencing factors, it is difficult to obtain standardised extracts and consistent qualities, so quality assurance of herbal medicines is definitely a complex problem that needs to be addressed.

1.1.4.2 Good Practices for total quality assurance

The current lack of uniform quality among herbal medicines and cases of adverse effects undermine consumers' and healthcare professionals' confidence. Herbal medicines are often marketed as dietary supplements, so their qualities are largely unregulated as compared to the stringent requirements necessary for synthetic

drugs. Fortunately, there are growing worldwide awareness and emphasis to regulate herbal medicines to ensure batch to batch consistency. To address the problems of variation in herbal medicines, a good total quality assurance system using a multi-pronged approach is needed. The quality assurance of synthetic drugs is mainly affected through production under good manufacturing practices (GMP) which is mandated by Food and Drug Administration (FDA). Similar to synthetic drugs, GMP regulations should also be imposed on botanical drugs [WHO, 2005]. However, GMP alone will not be sufficient to assure quality, due to the numerous factors and processes which can affect the chemical composition of botanicals.

The quality control measures should ideally start from the point of cultivation with good agricultural practices (GAP). GAP is a set of guidelines that requires botanical identification and regulation/ standardisation of every step involved in the cultivation process from field collection to post-harvesting processing, storage and transportation. The standards are not easily attainable as there are many steps to control. However, this is the first important step to ensure the quality of raw materials. GMP procedures employed for botanical products are similar to those used for the manufacturing of synthetic drugs, except for some special procedures and precautions specific for botanical products. In fact, there is increasing emphasis to ensure quality as WHO has published some general guidelines for GAP and GMP production of botanical products [WHO, 2003b; WHO, 2005a]. Besides GAP and GMP, good laboratory practices (GLP), good clinical practices (GCP), and good supply/ selling practices (GSP) are also needed [Cheng *et al*., 2006; Chan, 2005]. These are the broad guidelines for total quality assurance and the whole development process of herbal medicines into good quality products for patients' use.

1.1.4.3 Detection of contamination and identification of herbal medicines by chemical analyses

One aspect of quality control of herbal medicines includes testing for the presence of various contaminants such as micro-organisms, toxins, heavy metals, pesticides and synthetic drugs [Koh and Woo, 2000; Zou *et al*., 2006; Lau *et al*., 2003]. Regulatory control and guidelines on the analysis of some of these contaminants in botanical medicines/ Chinese Proprietary Medicines (CPMs) are available [Yee *et al*., 2005; Health Sciences Authority, 2006; WHO, 2005b].

Besides detection of contamination, another important role of quality control is to ensure the correct identity of botanicals. The methods which have been employed for identification and authentication of botanicals [Zhao *et al*., 2006] involve various taxonomic, physical, chemical and DNA molecular biology methods. Taxonomic identification using macroscopic, microscopic, organoleptic analyses have to be performed to assure the species identity and purity at various processing stages [Mahady *et al*., 2001]. These analyses may be subjective and highly dependent on the experience and skills of the evaluator to recognise the various types of medicinal plants and their microscopic features. This is increasingly difficult when the various species are very similar or when the substituted herb closely resembles the genuine material [Shim *et al*., 2005]. Dangerous errors in identification may occur such as misidentification and substitution of *Stephania tetrandra* resulting in renal failures (Section 1.1.2.3). In addition, it may not be sensitive enough for detecting small amounts of substitution or when there is a mixture of several herbs in the preparations. At times, the whole plant may not be available or the samples may be processed into final dosage forms (e.g. powder, tablets), thus rendering it difficult to identify by

morphological analysis [Yuan and Hong, 2003]. Alternative methods will be necessary in such cases.

Qualitative analysis of specific unique marker components using various chemical methods has been commonly used for species identification. It has the advantage of being able to analyse various types of dosage forms, mixture of herbs and different parts of the same species. However, identification based on markers requires knowledge of specific chemical characteristics of the medicinal herb. If the component is not unique in the species, it may be difficult to identify using a few chemical components and the results may not be conclusive. Furthermore, many of the intrinsic and extrinsic factors such as environment and development stages, conditions of cultivation, geographical sources, age and processing methods, may affect the chemical contents and marker components.

1.1.4.4 DNA fingerprinting

With the advancement of molecular biology, genetic or DNA fingerprinting of medicinal plants has been increasingly used for species identification. Unlike chemical identification, DNA analysis will not be affected by the variability in chemical components and it is non-tissue specific. It has been frequently used to identify the strain and origin of herbal medicines, to detect them at any phase of organism growth, as well as to differentiate them from adulterants/ substitutes or other species [Shim *et al*., 2005]. Unlike morphological analysis, it is less subjective and is suitable for closely resembling species. One limitation of DNA fingerprinting is that it will not differentiate different parts of the same plant and plants that have undergone different processing methods. It is usually done on fresh plant samples, as DNA is destroyed by harsh processing methods and is not intact in final dosage forms;

therefore making it unsuitable for the analysis of final herbal products and in-line process monitoring. Moreover, all the genotypic differences may not be translated into phenotypic differences in terms of chemical composition or contents and efficacy [Hong and Guo, 2006]. Therefore, chemical analysis is still needed to supplement the genetic information.

1.1.4.5 Standardisation

In addition to species authentication/ identification, quality control also involves standardising or controlling the contents of chemical components present in the botanicals to ensure consistent quality, safety and efficacy between batches. Some of the older analytical techniques include colorimetric and spectroscopic analyses which quantify the absorption of structurally related compounds at certain wavelengths. As several constituents may absorb at the same wavelengths, the methods are non-specific, and purification is needed before the analysis of a particular class of compounds, thereby their usage has been declining [Mahady *et al*., 2001].

In recent years, with the advancement of analytical techniques, chromatographic techniques which have powerful separation abilities, have been the methods of choice for the analysis of the wide array of chemical constituents [Mahady *et al*., 2001]. The common analytical and separation methods include thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), near infrared (NIR) and the various hyphenated methods such as liquid chromatography-mass spectrometry (LC-MS), GC-MS, LC-nuclear magnetic resonance (NMR), hyphenated capillary electrophoresis etc [Liang *et al*., 2004]. Hyphenated techniques showed improved performances in terms of elimination of

instrumental interferences, correction of retention time shift, increase selectivity, precision and separation abilities. They provide additional spectral information, which is helpful for qualitative analysis and structural elucidation [Liang *et al*., 2004].

Quality control using single active constituent is well established for synthetic pharmaceuticals. In general, a few pharmacologically active components are also widely employed for standardisation of the contents, and evaluation of the quality, authenticity, identity and quantitative chemical composition of medicinal herbs or preparations. For example, St John's Wort products are standardised to 0.3% w/w hypericin and *Panax ginseng* extract G115 has been standardised to 4% w/w total ginsenosides. However, there are many challenges facing standardisation of herbal medicines. A single herb often contains numerous natural components, so a combination of several herbs in herbal products may give rise to hundreds of components. This wide array of constituents makes the analysis more complex as compared to conventional drugs with only a single active compound. Since not all active components have been isolated, characterised or quantified, so many of their identities and activities remain unknown and many chemical standards are unavailable for quality control.

The choice of marker components that is representative of the whole botanical is difficult. For example, some researchers questioned [Fedec and Kolodziejczyk, 2000] the standardisation of St John's Wort based on hypericin, as other components also have antidepressant activities. In addition, the methods of extraction and sample preparation are also of importance in affecting the standardisation and quality control process, as the quantities of components varies with the methods of sample preparation. Although the quantities of a few markers are appropriate and consistent, it cannot be assumed that the quantities of other components are also consistent. As

the presence of multiple components may work together synergistically or complementarily to give therapeutic effects, so a few markers may not effectively represent the entire mixture. It does provide a criterion for quality control but may not be adequate to ensure therapeutic uniformity and the reliability of pharmacological and clinical research [Tyler, 1999]. Therefore, it is deemed necessary to develop quality control methods which regard the whole herbal preparation as a whole active 'compound' [Liang *et al*., 2004].

1.1.4.6 Chemical fingerprinting

In view of the above limitations of analysing a few components, in recent years, the use of chemical fingerprinting for the identification, standardisation and quality control of medicinal herbs has attracted a lot of interest. Chemical fingerprinting, which is a macro-analytical approach to evaluate the complex characteristics of medicinal herbs, usually utilises chromatographic methods (chromatographic fingerprinting) and the entire chromatogram's characteristics to achieve overall quality assessment and classification [Xie and Wong, 2005]. The chromatographic fingerprint is therefore a pattern of the chemical components in the extracts. Besides chromatography, other methods of chemical fingerprinting such as the use of modern Fourier transform infra-red spectroscopy (FT-IR) have also been established [Sun, 2006]. Instead of using only a few selected markers, fingerprinting is increasingly recommended and accepted as a better solution for quality control. In fact, chromatographic fingerprinting is one of the recommendations proposed by US FDA for botanicals [US FDA, 2004] and The European Agency for the Evaluation of Medicinal Products for herbal preparations [The European agency for the evaluation of medicinal products, 2000], as the fingerprints are unique and represent powerful

tools for the comparison, classification, identification and evaluation of samples. In Germany, the concept of 'phytoequivalence' was first developed [Tyler, 1999] to ensure consistency of herbal products. According to this concept, a chemical profile should be constructed and compared with the profile of a clinically proven reference product, and this is basically achieved by chemical fingerprinting methods.

Herbal medicines contain hundreds of unknown components of unknown activities and many of them are in low quantities. Moreover, there may be large variability within the same medicinal herb due to the various factors influencing quality (Section 1.1.4.1). Consequently, obtaining reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components is not an easy or trivial task. After obtaining the fingerprints with the various analytical techniques, including the hyphenated techniques, the challenge is to efficiently evaluate the obtained fingerprints and to use the information to address the problems of quality control. Visual methods have been used for qualitative comparisons of chromatograms. With the help of chemometrics, a newer discipline developed both in chemistry and statistics, research on the evaluation of the chromatographic fingerprints have been ongoing [Liang *et al*., 2004]. More research on the chemical fingerprinting methods and evaluation of fingerprints are necessary.

Since quality control is an essential prerequisite for ensuring their safety and efficacy, accurate assessment of quality is a critical step towards the widespread acceptance of herbal medicines. As seen from the above discussion, no single method is currently a perfect solution and multiple methods are usually needed for greater confidence of its quality [Liang *et al*., 2004]. Therefore, currently, one of the important challenges is to develop better methods and solutions for the quality assurance of herbal medicines.

1.2 Importance of antithrombotic and haemostatic therapies

1.2.1 Antithrombotic therapies

Cardiovascular diseases are leading causes of death and disability in the general world population, and more so amongst the elderly. Incidences have been rising due to unhealthy diets with high cholesterol food, affluent lifestyles and decreased physical activities. It is projected by WHO [WHO, 2003c] that cardiovascular diseases will be the leading killer in developed countries by 2010. Coronary heart diseases constitute one out of every five deaths in United States [American Heart Association, 2006]. In Singapore, for the past three years, ischaemic and other cardiovascular diseases contribute to the second while cerebrovascular diseases (including stroke) contribute to the fourth most common overall causes of death [Ministry of Health, 2005]. These diseases (contributing to 32% of total deaths) are also among the top ten conditions for hospitalisations [Ministry of Health, 2005]. Therefore, the continuous search for effective treatments for cardiovascular diseases is important, especially when the population is aging in many developed countries.

Blood platelet activation is fundamental to a wide range of physiologic and pathologic processes. The platelets play a critical role not only in normal haemostasis but also in thrombosis at damaged blood vessels or in regions of disturbed blood flow and blood stasis. Thrombus formation occurs through the inappropriate activation and aggregation of platelets. Arterial thrombi are formed under high shear conditions and are primarily composed of platelet aggregates held together by fibrin strands. The platelets also play a major part in both the initiation and growth of the venous thrombi, which are formed under low shear conditions and compose predominantly of fibrin and red cells. These evidences have led to postulations that platelet aggregation is a

major pathogenic mechanism in thrombosis, which leads to occlusion of blood vessels and ischemic injuries [Freson *et al*., 2006].

Aspirin, a non-steroidal anti-inflammatory drug (NSAID), is an example of an antiplatelet drug that is often used clinically to treat and prevent thrombotic events such as myocardial infarction, coronary heart disease, venous thromboembolism, deep vein thrombosis. It causes irreversible acetylation of hydroxyl group of the serine residue (Ser 530) in cyclooxygenase-1 within platelets, thereby preventing the biosynthesis of thromboxane A_2 (TxA_2), a potent vasoconstrictor and promoter of platelet aggregation [Vane and Botting, 2003]. As platelets are unable to regenerate cyclooxygenase, the effect remains for the lifespan of the platelet (8-10 days). However, the main drawback of long term usage of aspirin is the increase in the risk of gastrointestinal bleeding [Weisman and Graham, 2002]. Ticlopidine and clopidogrel are thienopyridine derivatives, belonging to another class of clinically used antiplatelet drugs, which covalently bind to platelet P2Y12 adenosine diphosphate (ADP) receptor and reduce platelet activation [Jacobson, 2004]. Ticlopidine, however, causes thrombocytopenia, neutropenia and aplastic anaemia. Clopidogrel has superseded ticlopidine as the drug of choice, but it causes diarrhoea and skin rashes. Dipyridamole, on the other hand, is a phosphodiesterase inhibitor which inactivates cyclic adenosine monophosphate (cAMP) and reduces activation of cytoplasmic second messengers. It also stimulates prostacyclin release and inhibits thromboxane A_2 formation, thereby inhibiting platelet function, but its effects may be short lasting and limited [Hankey and Eikelboom, 2003].

After the formation of primary haemostatic plug, the activated platelets facilitate the assembly of coagulation factors on the activated platelet membrane, which leads to generation of thrombin and the subsequent formation of fibrin around

the platelet aggregates, thereby stabilising the initial platelet plug. Therefore, the activation of blood coagulation cascade plays an etiological role in several thrombotic disorders such as unstable angina, deep vein thrombosis, disseminated intravascular coagulation. Two commonly used anticoagulant drugs used to treat such clinical conditions include heparin and warfarin. Heparin binds to antithrombin III (AT-III), which results in its enhanced affinity to several activated forms of factors, namely Factors IIa (thrombin), Xa and IXa. This binding renders the factors inactive. However, it has to be given parenterally, either intravenously or subcutaneously [Mueller, 2004]. Warfarin, an oral anticoagulant, is a coumarin which acts as an inhibitor of vitamin K epoxide reductase in the hepatocytes, thereby inhibiting the synthesis of vitamin K-dependent clotting factors such as II, VII, IX and X [Mueller, 2004]. However, warfarin has a narrow therapeutic index and haemorrhages are common complications when the plasma drugs levels are increased. Correct dosing and close monitoring of warfarin therapy are necessary. Another potential problem is it has many drug interactions with other drugs, herbs and even vitamin K-rich food, thus increasing the risk of adverse effects [Holbrook *et al*., 2005].

In view of the above, inhibitions of platelet function and coagulation pathways represent a very promising approach for the clinical prevention of thrombosis in pathological conditions. The current strategies that have been used to prevent or treat thrombotic disorders involve targeting the various steps in coagulation and platelet function. The thrombi are the source of thromboembolic complications of cardiovascular diseases such as arteriosclerosis, heart attacks, strokes, peripheral vascular diseases and angina, all of which represent the leading causes of morbidity and mortality. Therefore, with thrombosis playing the central role in the

pathophysiology of many cardiovascular diseases, the search for better antithrombotic medicines continues to be important.

Besides synthetic drugs, researchers have been looking for novel active compounds and active extracts from a wide variety of botanicals and dietary sources, especially when the adverse effects of herbal medicines may seem comparatively less severe than synthetic drugs. In view of the importance of antithrombotic therapy in cardiovascular diseases, medicinal herbs and their phyto-components that prevent thrombosis by inhibition of either platelet functions or coagulation pathways are, therefore, of special therapeutic interest. Several Chinese traditional herbs such as *Salvia miltiorrhizae*, *Ligusticum chuanxiong*, *Panax ginseng, Angelica sinensis,* have been traditionally said to have antithrombotic activities and are consumed to treat or prevent cardiovascular diseases [Zhu, 1998]. Recently, a comprehensive list of medicinal plants was found to be potential sources of lead compounds for antiplatelet and anticoagulant activities. Many examples of natural compounds in these medicinal plants/herbs and foods were identified as active antiplatelet or anticoagulant agents [Chua and Koh, 2006] and the search for good and safer novel antithrombotics is still ongoing.

1.2.2 Haemostatic therapies

Bleeding is an undesirable event which can occur after trauma, surgery, pathologic conditions or pharmacologic treatment with antiplatelet, anticoagulant, or thrombolytic therapies. When bleeding is the consequence of a specific defect of haemostasis, the goal of treatment is to correct the defect. For example, haemophilia is treated by transfusion of Factor VIII. Vitamin K is used for vitamin K deficiency bleeding and in warfarin overdoses. In very severe cases of bleeding, blood

transfusions are warranted. However, specific treatment may be impossible when bleeding is a result of multiple defects, or sometimes, when no specific cause can be identified. Non-transfusional drugs are important in such situations [Mannucci, 1998]. The haemostatic drugs used clinically include antifibrinolytic amino acids (aminocaproic acid and tranexamic acid), aprotinin, desmopressin, ethamsylate and conjugated estrogens [Mannucci, 1998]. Most of these drugs are used systemically to counteract severe cases of bleeding [White *et al*., 2000; White *et al*., 2001]. There are few established agents for less severe bleeding conditions. Furthermore, no effective topical haemostatic agent has been firmly established. Potential uses of externally applied agents may include traumatic injuries, bruises and external bleeding at incision site [White *et al*., 2000; White *et al*., 2001].

Other than synthetic drugs, several medicinal plants have been reported to have useful haemostatic effects. These haemostatic herbs such as *Panax notoginseng*, *Sophora japonica, Trachycarpus fortunei* and *Imperata cylindrical* [Zhu, 1998] may be used for internal and external bleeding/ haemorrhages. These include a broad range of haemorrhagic conditions such as vomiting blood (haematemesis), blood in urine (haematuria), blood in stools (haemafecia), uterine bleeding, excessive menstruation, and bleeding from trauma. Natural constituents from medicinal plants, for example dencichine, quercetin, have also been reported [Zhao and Wang, 1986; Ishida *et al*., 1989] to have haemostatic activities. These medicinal herbs may be used in less severe cases of bleeding. However, there is a relatively lack of information in this area and more research is needed to understand medicinal herbs and their natural constituents.

1.3 Medicinal plants as potential sources of novel therapeutic drugs

Plants have been utilised as medicines for thousands of years. According to WHO [WHO, 2003a], about 25% of modern synthetic medicines are made from or derived from medicinal plants that have been used traditionally. Drug discovery from medicinal plants has led to the isolation of early drugs such as digitoxin, aspirin, penicillin, quinine and morphine, of which most are still in use today. However, for many decades, synthetic and semi-synthetic drugs have been the main focus in drug discovery due to higher predictability and availability of established techniques such as molecular modelling, combinatorial chemistry and other new synthetic chemistry techniques. Comparatively, less information is known about medicinal plants and the drug discovery process from medicinal plants is more lengthy, costly and complicated.

In recent years, however, there has been rekindling of interest in the field of medicinal plants, as these plants provide a great diversity of new compounds. In fact, phytochemicals from medicinal plants are receiving greater attention in the scientific literature, in medicine and in the world economy. Medicinal plants continue to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain [Balunas and Kinghorn, 2005]. Paclitaxel from Pacific Yew tree, and vincristine and vinblastine from Madagascar periwrinkle, have been used for the treatment of various types of cancers. Irinotecan, topotecan, docetaxel and camptothecin are also examples of anticancer agents obtained originally from natural sources. Artemisinin, a sesquiterpene lactone, is an antimalarial compound discovered from *Artemisia annua*, while huperzine A from *Huperzia serrata* is currently in clinical trials for the treatment of Alzheimer's disease. Arteether, galantamine, nitisinone and tiotropium are also new medicinal plant-

derived drugs that have been recently introduced into the U.S. market [Balunas and Kinghorn, 2005].

The above successful examples showed the importance of medicinal plants for new drug discovery and have propagated more interests in this field. With such a large number of medicinal plants in the world and the large number of natural constituents present in each of these plants, this is definitely a very large resource for the search of new potential medicines. Therefore, more research should be done to discover the wide myriad of pharmacological activities from medicinal herbs and their phytochemicals.

1.4 *Pana*x species

1.4.1 Species of *Panax*

During the Han dynasty (206B.C. to 24A.D.), a plant known as 'sheng' was found and later known as ginseng, which literally means "man-root", due to its man-shaped figure. In 1883, the genus *Panax* was added to its name, which means "all healing" in Greek. The proponents of the "Doctrine of Signatures" believe that *Panax ginseng* is an all-healing man-root, and it is regarded as something of a panacea particularly in the East. *Panax ginseng,* together with the other *Panax* species, is later classified as one of the members of the plant family, Araliaceae [Sticher, 1998].

The root of *Panax ginseng* C. A. Meyer, commonly referred to as Chinese, Asian, or Korean ginseng, is one of the most established and earliest type of ginseng discovered. In fact, the annual market for this herb in United States is estimated to be about USD300 million [Mihalov *et al*., 2000]. *P. ginseng* can be processed into two forms, known as white and red ginseng. The former is the air or sun-dried root, whereas, the latter is steamed prior to drying or simply known as the steamed form of

fresh ginseng. The *P. ginseng* from Korea (Korean ginseng) usually refers to the steamed red root. Originally, *P. ginseng* is mainly grown in the wild in northeast China and Korea, especially in the mountainous areas. However, this wild-type ginseng is becoming less common and most of the commercial roots available in the market are the cultivated types of ginseng grown in large plantations [Tang and Eisenbrand, 1992; Court, 2000].

Besides *P. ginseng*, several *Panax* species grow in the northern hemisphere, from eastern Himalaya through China and Japan to North America. They can be divided into two groups depending on the shape of their underground parts [Sticher, 1998]. Group A contains carrot-like roots while Group B has long, horizontal rhizomes. *Panax quinquefolium* (American ginseng) and *Panax notoginseng*, which is indigenous to North America and northeast China respectively, belong to the same group (Group A) [Sticher, 1998] as *P. ginseng* and they are all well-known herbs used for different therapeutic purposes. Several researches have also been done on these two types of *Panax* species, in comparisons with *P. ginseng*. Examples of Group B include *Panax japonicum* (Japanese chikutsu ginseng), *Panax pseudoginseng* (Eastern Himalayas), and *Panax zingiberensis* (from southern China). There are still other *Panax* species and varieties [Court, 2000] such as *Panax vietnamensis* (Vietnamese ginseng), that have been discovered. In fact, more than ten *Panax* species reported in the world are valuable medicinal resources in traditional Chinese medicine and in folk medicine [Zhu *et al*., 2004a].

1.4.2 *Panax notoginseng* (Burk.) F. H. Chen

1.4.2.1 Introduction

The main medicinal herb of interest in this study is the dried root of *Panax notoginseng* (Burk.) F. H. Chen (abbreviated as *P. notoginseng*), also known as Sanqi or Tianqi. It belongs to the same family and genus as *P. ginseng*. It is mainly cultivated in the province of Yunnan in China. Southwestern parts of Wenshan in Yunnan are believed to produce the best quality of *P. notoginseng* in China [Dong *et al*., 2003]. *P. notoginseng* is a perennial herbage that bears compound leaves with lanced-shaped leaflets and clusters of greenish-yellow flowers [State Administration of Traditional Chinese Medicine, 1996]. In autumn, the flowers mature and develop into berries that turn red or red-black when ripened. The root is normally harvested in autumn before flowering, after three years of growth. After harvesting, the roots are cleaned, divided into main root, branch root ("Jintiao" "筋条") and rhizome ("Jiankou" "剪口"), and dried under the sun or with artificial heating at low temperatures [The State Pharmacopoeia Commission of People's Republic of China, 2000]. Sometimes, the roots (those commonly used in Singapore and Hong Kong) may be darkened with coal smoke and coated with insect wax to give a black shiny external appearance instead of its original greyish-brown appearance. *P. notoginseng* is divided into different traditional grades (called "tou" "头"in Chinese), which is ranked according to their dried size and weight. The "tou" means the number of individual roots in ~500 g, so the smaller the "tou", the greater is the size of the roots. It is traditionally believed that the bigger the size (or smaller "tou"), the higher is the quality [Dong *et al*., 2003].

1.4.2.2 Processing of *P. notoginseng*

Processing of Traditional Chinese herbs has a long history. Some of the processing methods used for Chinese herbs include cleaning the herb to remove unwanted materials, grinding, slicing, moistening, stir-frying with various agents, boiling and steaming. The reasons and significance of processing include reducing toxicity or side effects, potentiating the effects, changing the pharmacological properties, preserving active constituents, facilitating administration, increasing flavour or correcting the unpleasant taste, and increasing purity [Zhu, 1998]. For example, the cardiac toxicity of Aconitum species is due to aconitine, but it is hydrolysed to give non-toxic aconine after the herb is steamed. This process does not affect its anti-inflammatory and analgesic activities, which are due to other aconitine analogues e.g. pyro-type aconitine alkaloids [Zhu, 1998; Murayama *et al*., 1991]. Another example, raw *Rehmanniae glutinosae* is mainly used to purge heat and promote the generation of body fluids, but after processing, it has a warm property and becomes effective for enriching blood [Chan, 2005].

P. notoginseng is also available in two different forms—the raw and processed/ steamed form. Raw and steamed *P. notoginseng* are readily available as Chinese Proprietary Medicines (CPMs) (finished products such as tablets, capsules, powder, consisting of one or more ingredients) in the market. The raw roots can be processed by steaming or by stir-frying the roots in oil until the exterior is golden yellow [Yin and Guo, 1995]. There is very limited information regarding the exact procedures and conditions of steaming or frying, such as the equipments, temperature and duration used. Currently, there is no standard method of steaming raw *P. notoginseng*. The total amount of saponins was found to be greater in the steamed compared to the 'fried' and raw forms [Zhang, 1989]. Steaming is said to be a better

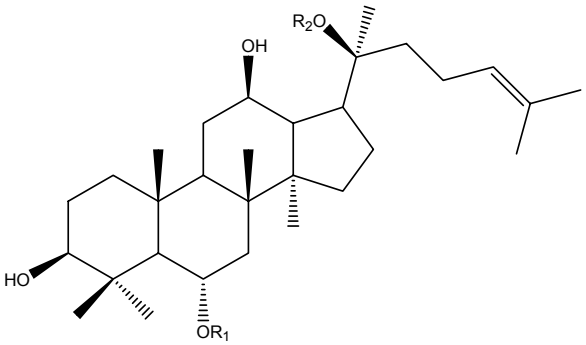
and preferred method of processing *P. notoginseng* as compared to frying which gave the lowest contents of saponins. *P. notoginseng* have also been stir-fried to different degrees by using different sizes of the roots [Tang, 1991]. The smaller roots were 'fried' to a greater extent and have lower saponin contents (60-70%) compared to the raw ones. The active components may differ greatly if products are not processed by the same method. Therefore, standardisation of the processing and manufacturing processes is important to ensure that herbal medicines have consistent quality and efficacy. There should be more detailed studies to further understand the effects of processing on the quality of *P. notoginseng*, in terms of individual saponins contents and the effects of steaming on efficacy.

1.4.2.3 Chemical constituents of *P. notoginseng*

The chemical constituents of this root are very complex, consisting of various saponins, amino acids, polyacetylenes, volatile oils, polysaccharides and flavonoids [Zhu, 1998; State Administration of Traditional Chinese Medicine, 1996]. The dammarane-type saponins account for 12% of the total root content and they are the main bioactive components of the root with a wide variety of activities [Zhu, 1998; Ma *et al*., 1999]. *P. notoginseng* has a much higher percentage of total saponins compared to *P. ginseng* and *P. quinquefolium*. The saponins include various ginsenosides and notoginsenosides. Many of these ginsenosides in the root are also present in other *Panax* species such as *P. ginseng* and *P. quinquefolium*. Although the *Panax* species share some common saponins, the total chemical profiles of the various species are different and needs to be carefully differentiated to prevent misidentification, accidental or intentional substitution with lower grades or cheaper species.

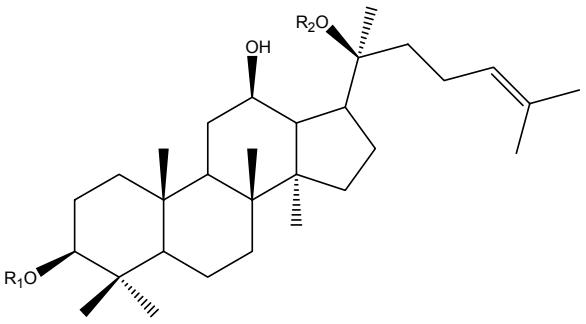
More than 30 ginsenosides have been isolated in the past few decades and more are still being discovered. The saponins can be hydrolysed to give the aglycones and sugar molecules. They are then classified into three main classes based on their aglycones—protopanaxadiol (e.g. ginsenosides Rb1, Rb2, Rc, Rd), protopanaxatriol (e.g. ginsenosides Rg1, Re, Rf) and oleanolic acid (e.g. ginsenoside Ro) (Figure 1.1) [Court, 2000]. Besides ginsenosides, another group of saponins termed as notoginsenosides, such as notoginsenosides R1 to R4, R6 to R9 [State Administration of Traditional Chinese Medicine, 1996; Zhou *et al*., 1981; Taniyasu *et al*., 1982; Matsura *et al*., 1983; Zhao *et al*., 1996], notoginsenosides A to N [Yoshikawa *et al*., 1997a; Yoshikawa *et al*., 1997b; Yoshikawa *et al*., 2001; Ma *et al*., 1999], notoginsenosides Fa, Fc and Fe [Yang *et al*., 1983], have also been isolated from *P. notoginseng*. Some of these were minor components present in *P. notoginseng*.

Dencichine (β-N-oxalyl-L-α,β-diaminopropionic acid), an important and bioactive amino acid, present in *P. notoginseng*, is known to have haemostatic activity [State Administration of Traditional Chinese Medicine, 1996; Zhang *et al*., 1990; Zhao and Wang, 1986; Long *et al*., 1996]. At least 16 types of amino acids, with seven being essential amino acids such as aspartic acid, glutamic acid, arginine, lysine, leucine, were found and the total amino acids content is about 7.73% [State Administration of Traditional Chinese Medicine, 1996]. Trilinolein, a triacylglycerol with three linoleate residues, is another important component found to have protective effects against cardiovascular diseases by reducing thrombogenicity, arrhythmias, increasing erythrocyte deformability and counteract free radical damages [Chan *et al*., 2002]. Polyacetylenes such as panaxynol (falcarinol), panaxydol, 1,8-heptadecadiene-4,6-diyne-3,10-diol, panaxytriol (falcarintriol) have also been discovered in *P. notoginseng* and found to have activities such as cytotoxic, antiplatelet and anti-



Protopanaxatriol (R_1, R_2 = H)

Saponins	R_1	R_2
Ginsenoside Rg1	-Glc	-Glc
Ginsenoside Re	-Glc^{2}-^{1}Rha	-Glc
Ginsenoside Rf	-Glc^{2}-^{1}Glc	-H
Notoginsenoside R_1	-Glc^{2}-^{1}Xyl	-Glc
Notoginsenoside R_2	-Glc^{2}-^{1}Xyl	-H
Notoginsenoside R_3	-Glc	-Glc^{6}-^{1}Glc
Notoginsenoside R_6	-Glc	-Glc^{6}-^{1}Glc (α)



Protopanaxadiol (R_1, R_2 = H)

Saponins	R_1	R_2
Ginsenoside Rb1	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Glc
Ginsenoside Rb2	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Ara(p)
Ginsenoside Rb3	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Xyl(p)
Ginsenoside Rc	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Ara(f)
Ginsenoside Rd	-Glc^{2}-^{1}Glc	-Glc
Notoginsenoside R_4	-Glc^{2}-^{1}Glc	-Glc^{6}-^{1}Glc^{6}-^{1}Xyl
Notoginsenoside Fa	-Glc^{2}-^{1}Glc^{2}-^{1}Xyl	-Glc^{6}-^{1}Glc
Notoginsenoside Fc	-Glc^{2}-^{1}Glc^{2}-^{1}Xyl	-Glc^{6}-^{1}Xyl
Notoginsenoside Fe	-Glc	-Glc^{6}-^{1}Ara(f)

Figure 1.1. Chemical structures of some saponins. Abbreviations: Glc, glucose; Ara(f), arabinose in furanose form; Ara(p), arabinose in pyranose form; Rha, rhamnose; Xyl, xylose.

inflammatory effects [Washida and Kitanaka, 2003]. Yoshikawa *et al*. [1997b] isolated an acetylenic fatty acid glycoside known as notoginsenic acid beta-sophoroside, from the roots. Polysaccharides such as sanchinan-A, arabinogalactan, extracted from *P. notoginseng* are considered to be active immunostimulatory constituents [State Administration of Traditional Chinese Medicine, 1996; Gao *et al*., 1996]. Detailed characterisation of the polysaccharides from *P. notoginseng* root cell walls showed that they include cellulose, pectic polysaccharides such as homogalacturonan and rhamnogalacturonan, and non-cellulosic polysaccharides [Zhu *et al*., 2005]. A small chitinase-like antifungal protein with a molecular weight of 15 kDa was also isolated from the root [Lam and Ng, 2001a]. Moreover, the root contains a series of sesquiterpenes essential oils/ volatile oils and some of the major volatile components have been identified as α-guaiene, β-guaiene, and octadecane [Zhu, 1998]. Some other components include flavonoids such as quercetin, kaempferol, and common plant sterols such as β-sitosterol, stigmasterol, daucosterol [Yin and Guo, 1995].

With regards to comparisons of chemical constituents between raw and steamed *P. notoginseng*, only one report has been found. Yang *et al*. [1985] have isolated some saponins from both raw and processed *P. notoginseng* using open column chromatography and identified some of the saponins. It was found that the yields of ginsenosides Rb1, Rd, Re, Rg1, Rh1 and notoginsenosides R1 and R4 were lower in the steamed form as compared to the raw form. Ginsenosides Rg2 and Rg3 were obtained from processed herb but not from the raw form. The saponins may be thermolabile. Therefore, more studies are needed to understand the differences between raw and steamed *P. notoginseng* using advance analytical techniques.

Since *P. notoginseng* contains some common constituents as *P. ginseng*, the stability and possible changes in the ginsenosides content of *P. ginseng* after heat processing may provide some insights on the possible changes in *P. notoginseng*. White and red ginseng (produced by steaming raw ginseng) have been compared [Kim *et al*., 2000; Kwon *et al*., 2001]. Kim *et al*. [2000] found some differences in chemical constituents and pharmacological activity between them. Ginsenosides F4, Rg3 and Rg5, which were not present in raw *P. ginseng*, were produced after steaming, with ginsenosides Rg3 and Rg5 being the most abundant ginsenosides in steamed ginseng. White ginseng was found to contain panaxynol and panaxydol while red ginseng contains, in addition to the two components, heptadec-1-ene-4,6-diyne-3,9-diol and panaxytriol [Kitagawa *et al*., 1987]. The degradation of ginsenosides in *P. quinquefolium* during microwave and conventional heating (heating using water bath) was investigated [Ren and Chen, 1999]. Malonyl ginsenosides (m-Rb1, m-Rc, and m-Rd) were much less stable and readily demalonylated on heating, with 3 to 60 times higher rate constant value than that of the neutral ginsenosides (e.g. Rb1, Rc, Rd, Re). Interestingly, at the same temperature, the effect of microwave heating on the degradation of ginsenosides was the same as that of conventional heating, so there were no significant differences between the heating methods. Although there is some information on the chemical changes in *P. ginseng*, there is still a need for studies in *P. notoginseng* as the chemical profiles between species are different and the changes may not be completely inferred from *P. ginseng*.

1.4.2.4 Pharmacological studies of *P. notoginseng*

The two forms of *P. notoginseng* have different traditional uses. The raw form is officially recognised in Chinese medicine for its haemostatic and cardiovascular

properties, to arrest bleeding, disperse blood clots, improve blood circulation, disperse bruises, cause subsidence of swelling and alleviate pain [Zhu, 1998; The State Pharmacopoeia Commission of People's Republic of China, 2000 and 2005]. Interestingly, promoting circulation and haemostasis seems to be of opposite effects scientifically, but this may reflect the theory of Traditional Chinese Medicine whereby there are opposing factors to maintain overall physiological balance. On the other hand, the steamed *P. notoginseng* has been claimed to be more of a tonic used to "nourish" blood, to increase production of various blood cells in anaemic conditions [State Administration of Traditional Chinese Medicine, 1996] and it is traditionally said to be used in 'weaker' patients who cannot tolerate the more 'potent' raw form. Due to the different pharmacological actions and clinical indications of raw and steamed *P. notoginseng*, using the wrong form of herb may lead to undesirable results for the patients or consumers. Differentiating between the two forms is therefore important.

Currently, most of the investigations are focused on raw *P. notoginseng*, as this is more commonly used for therapeutic purposes. Its well-known haemostatic effect has been studied scientifically [White *et al*. 2000; White *et al*. 2001; Fan *et al*., 2005]. Besides the above effects, it also showed several beneficial cardiovascular effects. Animal studies have shown that *P. notoginseng* extracts decreased blood pressure and peripheral vascular resistance. It increases coronary blood flow, treats experimental myocardial ischemia, alleviates angina pectoris, increases cardiac contractility, may have anti-arrhythmic effects and helps in craniocerebral trauma [Zhu, 1998; Tang and Eisenbrand, 1992]. Other reported pharmacological activities of raw *P. notoginseng* extracts and its total saponins (*P. notoginseng* saponins, PNS) include reduction of blood viscosity, inhibition of platelet aggregations [Ma and Xiao,

1998; Liao and Li, 1997; Wang *et al*., 2004; Su *et al*., 1996], anti-inflammatory and analgesic effects [Zhu, 1998; Tang and Eisenbrand, 1992; Rhule *et al*., 2006], retardation of the progress of early diabetic nephropathy [Lang *et al*., 1998], immunological adjuvant activities [Sun *et al*., 2006], prevention of liver fibrosis and hepatic microvascular dysfunction [Park *et al*., 2005]. Furthermore, anti-carcinogenic and anti-proliferative activities have been reported in the roots of *P. notoginseng* [Konoshima *et al*., 1999; Lam and Ng, 2001b]. Notoginsenoside R1, one of the distinct saponins present in *P. notoginseng*, has been found to increase the fibrinolytic potential in cultured endothelial and smooth muscle cells [Zhang *et al*., 1997a; Zhang *et al*., 1995], to counteract endotoxin-induced activation of endothelial cells [Zhang *et al*., 1997b] and to inhibit TNF-alpha-induced PAI-1 production in human aortic smooth muscle cells [Zhang and Wang, 2006].

Few scientific studies have been done on steamed *P. notoginseng*, so very few researchers compared the pharmacological actions of the two different forms. One such comparative study [Zhang *et al*., 1989] found that the total saponins from raw *P. notoginseng* caused a greater increase in protein synthesis compared to processed *P. notoginseng*. Chen *et al*. [1984] showed that steamed *P. notoginseng* increased the cholesterol and triglycerides in rats while raw *P. notoginseng* can reduce the cholesterol slightly. The traditional uses of both forms are on the haematological and cardiovascular system. However, there are currently no scientific evidences on their pharmacological differences on the haematological system. Therefore, more studies are needed to provide evidences and understanding of the specific differences between raw and steamed *P. notoginseng*.

As some of the *P. notoginseng* saponins are present in *P. ginseng*, some information can be gained from the extensive literature which studied the

pharmacology of some common saponins individually. It was found that the bioactivities of ginsenosides varied depending on different types of aglycones, various sugar moieties and stereoisomerism [Attele *et al*., 1999]. One study revealed that ginsenosides with protopanaxadiol type aglycone have neurite outgrowth activity on human neuroblastoma cells, while other types of ginsenosides have no effect [Zhu *et al*., 2004a]. Within the same herb, the pharmacological actions of the components may also work in opposition. For example, ginsenoside Rg1 is central nervous system (CNS)-stimulative while ginsenoside Rb1 is CNS-sedative [Shibata, 2001]. Another ambiguity in vascular pathophysiology was revealed by the evidences that predominance of ginsenoside Rg1 led to angiogenesis for wound healing, while a predominance of ginsenoside Rb1 inhibited angiogenesis and prevented chemoinvasion [Sengupta *et al*., 2004]. A wide range of pharmacological actions including CNS, cardiovascular, cholesterol-lowering, immunomodulatory effects, have been reported in individual ginsenosides [Shibata *et al*., 2001]. Therefore, this large diverse class of saponins are often regarded as the main bioactive components in the *Panax* species. Antitumour activities were reported for some saponins [Attele *et al*., 1999; Shibata, 2001], especially those components and fractions obtained from heat-processed ginseng or red ginseng [Keum *et al*., 2000; Yun *et al*., 2001a; Park *et al*., 2002a]. Therefore, the ginsenosides demonstrated ability to target a myriad of tissues. Since the components produce effects that are different from each other and a single component can initiate multiple actions, these may account for the complexity of the overall pharmacology of *Panax* species and more work is needed to understand the different activities of the various constituents.

1.4.2.5 Quality control of *P. notoginseng* and its related species

As discussed in Section 1.1.4, quality control involves identification and authentication of the plant, as well as determination of its chemical contents to ensure consistent qualities. Therefore, this section gives an overview of the existing DNA fingerprinting, chemical fingerprinting methods and chemical assays that have been specifically used for the quality control of *P. notoginseng* roots and its products [Lau and Koh, 2006].

DNA fingerprinting and molecular biological methods

Some of the *Panax* species are very similar macro-morphologically and microscopically, making them difficult to differentiate and prone to misidentification and substitution. One approach will be the use of genetic methods for more objective identification of the individual species. A diverse array of DNA-based marker technologies has been established to explore various DNA polymorphisms [Shaw *et al*., 2002].

Arbitrarily-primed polymerase chain reaction (AP-PCR) and Randomly amplified polymorphic DNA (RAPD) techniques have been applied [Shaw and But, 1995] to differentiate three *Panax* species from one another and their common adulterants. *P. ginseng* is more closely related to *P. quinquefolium* than to *P. notoginseng*. Both methods were equally successful but the amount of DNA required for RAPD was 10-fold less than AP-PCR and the primers for RAPD were commercially available. Six *Panax* species and two common adulterants [Ngan *et al*., 1999] were successfully differentiated using restriction fragment length polymorphism (RFLP). Using different enzymatic digestion, more than one distinctive RFLP profiles can be generated, thus providing more markers for

identification. 5S-rRNA spacer domains were also isolated [Cui *et al*., 2003] from *P. notoginseng* and other *Panax* species. The spacer domains showed 75% DNA identity among all *Panax* species, but not the adulterants. Multiplex amplification refractory mutation system [Zhu *et al*., 2004b] allowed authentication of five *Panax* species with simultaneous detection of four sites of nucleotide differences on two completely different genes. Identifying a herb within a complex matrix of numerous herbs in the final products is challenging. However, a ginseng marker primer (SIM2) that specifically amplified a fragment from the DNA of *Panax* species, was identified [Shim *et al*., 2005]. The gradient PCR method using the SIM2 primer was used to uniquely identify *Panax* species in herbal preparations containing diverse herbal components. The study suggests the possibility of developing a *Panax* species identification kit for medicinal plants and their preparations. The technique may be applied to other herbs and a new simultaneous identification method of herbal medicines may be possible in the future.

Chemical assays and fingerprinting

Besides differentiating between different species, it is important to determine the quality within each species. This difference in quality in terms of chemical contents, however, may not be detected using DNA fingerprinting methods. These chemical components may be directly related to the safety and therapeutic efficacies of the herbal medicines. Therefore, chemical analysis and chemical fingerprinting would be needed to supplement the information provided by DNA fingerprinting (Section 1.1.4.4).

Compared to the analysis of *P. ginseng* and *P. quinquefolium*, reports describing the methodology for the quality control of raw *P. notoginsen*g were less

abundant. Older studies employed TLC with densitometry [Zhou and Zhang, 1981] for the analysis of saponins. HPLC methods are now the most commonly used methods for their quality control. The qualitative profiling of the raw *P. notoginseng* roots compared to other *Panax* species has been reported [Zhou *et al*., 2001; Zhai *et al*., 2001]. The ratios of ginsenoside Rg1 to Re is different for *P. ginseng*, *P. quinquefolium* and *P. notoginseng*. Furthermore, HPLC fingerprints of *P. notoginseng* were analysed using cluster analysis, allowing classification of the roots into four different qualities [Wang and Bi, 2003].

Due to the complexity of the chemical components and the similarity of numerous saponins, the simultaneous analysis of all saponins is difficult. Wang *et al*. [2000a] and Jiang *et al*. [2000] quantified up to three saponins present in the raw herbs using HPLC. Another HPLC-UV method [Li *et al*., 2005] simultaneously quantified six major saponins in another 23 *P. notoginseng* samples. Chuang *et al*. [1995] has managed to analyse twelve ginsenosides in *P. notoginseng* and compared them to other *Panax* species. The saponin contents in *P. notoginseng* and *P. quinquefolium* were generally higher than *P. ginseng*. Using the chemical data and external appearance, the quality and origin of the herb can be postulated. Similarly, Yamaguchi *et al*. [1988] analysed twelve acidic and neutral saponins, namely, ginsenosides Ro, mRb1, mRb2, mRc, mRd, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and found differences in their contents in five *Panax* species. *P. notoginseng* did not contain saponins of the oleanolic acid prototype unlike *P. ginseng* and *P. japonicus*.

Ginsenosides in raw *P. notoginseng* were also quantified using HPLC-evaporative light scattering detector (ELSD) [Li and Fitzloff, 2001; Wan *et al*., 2006]. ELSD, a mass detector, was found to be a suitable detector for saponins and it can quantify the saponins proportionally. Twenty-three *P. notoginseng* samples collected

from different locations were analysed by HPLC-ELSD [Sha and Zhang, 2005] and it was found that there was no absolute linkage between the content of saponins and the traditional grade of the root. A comprehensive study [Zhu *et al*., 2004a] involving 47 samples from 12 *Panax* taxa was conducted. Eleven ginsenosides were quantified by HPLC to characterise the chemical constituent pattern of each *Panax* species and their relationship with the genetic varieties. Each *Panax* taxon showed its own characteristic chromatographic profile, which also appeared as a specific shape in an 11-direction radar graph constructed on the basis of the quantitative results. The 12 *Panax* species were grouped into two main groups based on their components. Different grades (called "tou" "头", Section 1.4.2.1) of *P. notoginseng* were analysed. Results showed that there was a small increase in saponin content with the increase in size or grades, but the differences were not significant. The absence of oleanolic acid saponins also distinguished this taxon from the other *Panax* species [Zhu *et al*., 2004a].

In another study, using HPLC and spectrophotometry, four saponins, dencichine, flavonoids and polysaccharides in raw *P. notoginseng* roots from different regions of growth in China, as well as from different seasons of harvest and market grades, were analysed separately [Dong *et al*., 2003]. This is one of the few detailed studies on the regional and seasonal variations of its chemical constituents. It was found that the roots from southwestern parts of Wenshan were of the best quality and the best season for harvest was September to October. The unseeded plants (flower-picked plants) also produced a better quality. Being an active haemostatic constituent in *P. notoginseng*, dencichine can also serve as a quality control marker. Unlike saponins that differ greatly in roots obtained from different sources, the contents of dencichine in *P. notoginseng* roots from different regions and seasons did not vary

significantly [Dong *et al*., 2003]. These results provided useful information for the quality control and development of GAP standards for *P. notoginseng*.

Capillary electrophoresis (CE), specifically, micellar electrokinetic chromatography (MEKC), has been developed for determining saponins in *P. notoginseng*. Its sensitivity permitted the analysis of minor saponins such as ginsenosides Rh1 and Rg2 [Wang *et al*., 2006]. Besides saponins, proteins from *P. notoginseng* have been used to distinguish it from other varieties and these were also analysed using CE [Hou *et al*., 2000].

LC-MS, being a sensitive and specific analytical technique, is increasingly used for a wide variety of applications including authentication and analysis of complex samples. *P. ginseng* and *P. quinquefolium* can be differentiated based on their ginsenosides using LC-MS techniques. LC-MS-MS [Kite *et al*., 2003] with negative ion electrospray conditions was used to profile the malonylated and acetylated ginsenosides in *P. notoginseng*, *P. ginseng* and *P. quinquefolium*. Analyses revealed different profiles of malonyl-ginsenosides in the three *Panax* species and this method assisted in the quality control of *Panax* species. LC-MS was also employed to identify *P. notoginseng* roots and its presence in complex Chinese patented medicines containing other herbs such as *Salvia miltiorrhiza* (Danshen) [Xiao *et al*., 2004; Zhang and Cheng, 2006]. Recently, 'Xuesetong' injection, a CPM consisting of total saponins from *P. notoginseng*, has been analysed using HPLC-ESI-MS/MS methods and the large variability of contents among ten samples was shown [Lai *et al*., 2006].

Besides the widely used chromatographic methods, near infrared spectroscopy [Chen and Sorensen, 2000] has been demonstrated to have the potential for rapid quality control of crude herbal plant materials such as *P. notoginseng*. The results obtained were satisfactory for the classification of crude herbs into low, medium and

high qualities and the technique is non-destructive. The main drawback of this method is the calibration step, which requires analyses of several natural samples covering the spectral variation of the samples.

Although most work has been carried out on the non-volatile components of the *Panax* species, fingerprinting of semi-volatile components also provided good differentiation of the various species [Shellie *et al*., 2003]. Comprehensive two-dimensional gas chromatography (GC x GC) chromatograms revealed the presence of numerous common components and some species-specific components. Furthermore, the use of GC x GC-quadrupole mass spectrometry permited the identification of some major components [Di *et al*., 2004].

Novel immunochemical methods have been proposed for the analysis of *Panax* species. An interesting combination of Enzyme-linked immunosorbent assay (ELISA), western blotting and immunoaffinity concentration using an anti-ginsenoside Rb1 monoclonal antibody was used for the qualitative and quantitative analysis of ginsenosides in *Panax* species and traditional Chinese herbal medicines [Fukuda *et al*., 2000]. Recently, the authors have also developed new monoclonal antibodies against the protein components of *P. ginseng* and a combination of ELISA, RAPD, and TLC with blotting and immunostaining methods, enabled authentication of four different *Panax* species including *P. notoginseng* [Tanaka *et al*., 2006]. Although these immunochemical methods are specific for individual chemical components and showed good correlation with HPLC methods, but they may be too elaborate for simple routine analysis of samples.

A variety of genetic and chemical methods have been used for the quality control of *Panax* species, involving both the identification/ authentication of the correct species as well as the quantification and standardisation of its chemical

constituents. From the above chemical studies, the quantitative results showed a general lack of consistent quality among different *Panax* species. Due to the many factors influencing quality, total quality management is definitely complex and is an uphill task. Therefore, a combination of different complementary technologies or methods is usually needed.

CHAPTER 2

HYPOTHESES AND OBJECTIVES

The quality of herbal medicines is a critical and challenging issue that needs to be addressed to ensure their safety and efficacy. The importance of quality has been extensively discussed in Section 1.1.4. More research is needed to develop better methods to ensure the quality of herbal medicines, to understand their chemical compositions and to explore the scientific basis for their traditional uses.

The root of *P. notoginseng* is an important Chinese medicinal herb with a long history of usage. Its processing, chemical constituents, pharmacological actions and current quality control are reviewed in Section 1.4.2. The raw and steamed forms have different traditional uses. It is important to understand the chemical and biological changes upon steam processing and to develop methods for quality control so as to safeguard the interests of patients consuming herbs and herbal products. Due to the above reasons and the general lack of scientific studies comparing raw and steamed *P. notoginseng*, *P. notoginseng* is thus selected as the main herb of study in this thesis.

Chemical assays and chromatographic fingerprinting are useful quality control measures. Most of the previous analytical studies focused on other *Panax* species, especially *P. ginseng*, while for those studies on *P. notoginseng*, only the raw form of *P. notoginseng* has been studied (Section 1.4.2.5). DNA fingerprinting for the identification and authentication of *Panax* species has been reported. However, DNA fingerprinting is not able to detect the effects of processing on the samples as steam treatment destroys the DNA. There are very few reports on the effects of processing in this species. This general lack of quality control and analytical methods for raw and steamed *P. notoginseng* makes it difficult for further research on this root, as safety and efficacy studies will require validated analytical methods to ensure that the

studies are reproducible and the qualities are consistent. To date, the differences in chemical composition of raw and steamed *P. notoginseng* are still not clearly understood. Although there are some chemical studies on the differences between white and red (steamed) *P. ginseng*, the results may not be directly extrapolated to *P. notoginseng*, as different species have different chemical compositions. The potentially unique chemical components may serve as important marker compounds for identifying steamed *P. notoginseng*. Most research has focused on the bioactive saponins (e.g. ginsenosides and notoginsenosides) present in these *Panax* species. Besides saponins, dencichine (Section 1.4.2.3), a haemostatic agent, is another important component present in *P. notoginseng*. Although one of the traditional indications for raw *P. notoginseng* is haemostasis, but this effect has not been indicated for the steamed form, and it is not known what the contributing factors are or whether dencichine plays a role in this particular difference in their indications, therefore studying this component may provide a better understanding of their differences.

As discussed in Chapter 1, botanicals are potential sources for novel therapeutic drugs and there is still a wide myriad of pharmacological activities to be discovered. Moreover, antithrombotic and haemostatic therapies are important classes of drugs where ongoing search for good alternatives and potential drugs is still needed. *P. notoginseng* has been traditionally used for haemostasis and cardiovascular diseases (Section 1.4.2.4). It may be promising as an alternative antithrombotic. To date, there are not many studies on the anticoagulant activities of *P. notoginseng* roots. Although the pharmacological effects of raw *P. notoginseng* roots have been relatively well studied, studies are lacking for the steamed form, especially with regards to its haematological effects and on the difference (if any) in activity between

raw and steamed form. With modernisation of herbal medicines, it is important to provide clear scientific evidences for efficacy to support their traditional uses. Furthermore, it is of interest to see if differences in chemical components may result in any differences in biological activities. To date, few studies investigated both the antiplatelet and anticoagulant effects of individual chemical components (Section 1.4.2.4). Besides, most of the haematological studies were done on the common saponins present in *P. ginseng* (e.g. ginsenosides Ro, Rg1, Rg2, Rg3, Rb1 and panaxatriol). There are still many components that have not been tested for haematological effects. Therefore it is of interest to study their activities.

Hypotheses

In view of the previous literature, it is hypothesised that processing of raw *P. notoginseng* roots by steaming changes the concentration and composition of chemical constituents in *P. notoginseng* and that the chemical profiles of the raw and steamed forms can be differentiated from each other and from its closely related species, using various qualitative and quantitative methods.

Furthermore, it is hypothesised that with the changes in chemical constituents upon steaming, there may be changes in the biological activities (namely, platelet aggregation, blood coagulation and haemostasis).

Objectives

In order to test the above hypotheses, the overall objectives of this study are to develop methods for the quality control of *P. notoginseng*, and to study the effects of processing on the chemical and biological differences between raw and steamed *P. notoginseng*. To achieve this, the current study is designed to meet the following specific objectives:

1. To develop novel and validated HPLC methods for the quality control of *P. notoginseng* and its related *Panax* species, to study the effects of processing on the various saponin concentrations in *P. notoginseng* and to quantitatively compare the chromatographic fingerprints of raw and steamed *P. notoginseng*.
2. To isolate and identify the main unknown marker components present in steamed *P. notoginseng*, and to develop a novel and validated LC-MS/MS method for the analysis of dencichine in raw and steamed *P. notoginseng* samples and its related species.
3. To investigate the effects of the different forms of *P. notoginseng* and its related species on the haematological system, specifically, on blood coagulation pathways and platelet aggregations in both *in vitro* and *ex vivo* models, and their *in vivo* haemostatic effects.
4. To investigate the activities of chemical components of *P. notoginseng* on blood coagulation and platelet aggregations *in vitro*, so as to understand the components that are contributing to the activities and to identify potential leads for antithrombotic drugs.

CHAPTER 3

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF *PANAX NOTOGINSENG* AND RELATED SPECIES

In this chapter, novel HPLC analytical methods for the quality control of *P. notoginseng* and its related *Panax* species, are developed and validated. This chapter consists of 2 parts. Firstly, a novel HPLC method for the chemical fingerprinting and analyses of saponins is developed. The validated method is applied to *P. notoginseng* from various sources, other *Panax* species and *P. notoginseng* products. It is also applied to raw and steamed *P. notoginseng* to study the effects of processing on the chemical components (Section 3.1). Secondly, a quantitative pattern matching method is optimised and successfully used for the comparisons of whole chromatograms of raw and steamed *P. notoginseng* samples (Section 3.2).

3.1 Chemical fingerprinting and analyses of saponins

3.1.1 Introduction

As discussed in Chapter 1, it is important to differentiate raw and steamed *P. notoginseng* due to their different traditional indications. Misidentification of the samples may lead to undesirable effects or inefficacy. Saponins (e.g. ginsenosides and notoginsenosides) are the main class of bioactive compounds in *Panax notoginseng* and several of them have rather similar structures or are isomers of each other. Due to the complexity of the chemical components and the similarity of numerous saponins, the analysis of *P. notoginseng* and its related species is a challenge. With the advancement of newer technologies, there are currently many methods such as TLC,

HPLC, GC, CE, IR and immunochemical methods, which can be employed for the analysis of ginsenosides that were mainly in *P. ginseng* [Fuzzati, 2004]. Sensitivity, separation, resolution and precision are some important criteria to consider. There are several guidelines for the validation of analytical methods of synthetic drugs [International conference on harmonisation, 1996; EURACHEM, 1998], but there are not many validated analytical methods for the analysis of herbal samples such as *P. notoginseng*.

Although there are some reports of methodology for the quality control of raw *P. notoginseng* (Section 1.4.2.5), however, to date, no extensive analytical study on steamed *P. notoginseng* was found. In fact, to date, only one study [Yang *et al*., 1985] estimated the concentrations of some saponins from raw and processed *P. notoginseng* using the yields obtained from simple open column chromatography. There are no further detailed analytical or comparative studies on the qualitative and quantitative analysis of both raw and steamed *P. notoginseng* using analytical techniques such as HPLC, GC-MS or LC-MS^n. The existing HPLC methods for raw *P. notoginseng* were not satisfactory for the analysis of steamed *P. notoginseng*, due to the presence of numerous saponins of lower polarity than those in the raw herb. These saponins may not elute out or separate using existing methods.

The objective of this section is to develop novel and validated HPLC method for the quality control of *P. notoginseng* and its related *Panax* species and to study the effects of processing on the various saponin concentrations in *P. notoginseng*. HPLC with UV detection, being one of the most commonly used methods in routine quality control, will be employed in this study to qualitatively and quantitatively analyse *P. notoginseng*. Qualitative comparisons of chromatograms are carried out visually and by hierarchical clustering analysis (HCA). In addition to qualitative comparisons,

simultaneous quantification and comparison of the concentration of six main saponins in raw *P. notoginseng, P. ginseng* and *P. quinquefolium* samples obtained from several different sources will be carried out. The saponin concentrations of individual *P. notoginseng* roots collected from a good agricultural practice (GAP) farm will be determined to study their variation. Lastly, the effects of steaming on the chemical profiles, and the saponin concentrations in raw and steamed *P. notoginseng* will also be investigated [Lau *et al*., 2003; Hong *et al*, 2005].

3.1.2 Experimental

3.1.2.1 Materials

The solvents used were of HPLC grade. The water used was treated with a Milli-Q water purification system (Millipore, Molsheim, France). Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 standards were purchased from Indofine Chemical Company (Somerville, NJ, USA). Notoginsenoside R1 was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ginsenoside Rb3 was obtained from Delta Information Centre for Natural Organic Compounds (China).

The raw *P. notoginseng* roots were obtained from six different Chinese medical shops/ sources in Singapore (Samples 1 to 3) and China (Samples 4 to 6). Dried raw *P. notoginseng* roots, which were three-year-old and graded as 30 'tou' (about 30 roots in 500 g), were randomly collected and pooled from a good agricultural practice (GAP) farm in Wenshan (Wenshan Miaoxiang Notoginseng Industry Corporation, Yunnan, China) (Sample 7). The reference standard raw *P. notoginseng* (Sample 8) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Three different quality

grades of *P. notoginseng* sample 1, 40 'tou' (Sample 1-40), 80 'tou' (Sample 1-80), 120 'tou' (Sample 1-120), were obtained from a medical shop in Singapore. Twelve individual *P. notoginseng* roots (labelled as roots WS-1 to WS-12), which were three-year-old and graded as 30 'tou', were separately collected from the single farm in Wenshan (Wenshan Miaoxiang Notoginseng Industry Corporation, Yunnan, China). Furthermore, two samples of two-year-old roots (Y2-1 and Y2-2) in the same farm were randomly collected from the same shaded plot and dried in the oven at low temperatures of 40-45°C. A *P. notoginseng* injection (50 mg/ml), containing *P. notoginseng* saponins (PNS), was obtained from Yick-Vic Chemicals and Pharmaceuticals Ltd (Hong Kong).

Eleven pairs of raw and steamed *P. notoginseng* products (CPMs) consisting of only one herb, were bought from various local Chinese medical shops. Products from the same manufacturer but in different dosage forms were considered as separate products. The products are listed in Table 3.1.

Samples of *P. ginseng* and *P. quinquefolium* roots were obtained from several different medical shops in Singapore. A Korean red ginseng was obtained from Pungki Ginseng Cooperative Association (Kyoungsangbuk-do, Korea). The reference standard red ginseng (*P. ginseng*) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The photos of the different species are shown in Figure 3.1.

Table 3.1. List of *P. notoginseng* CPMs that were analysed

Pair no.	Sample code	Sample name	Brand
1	1R	Pure Raw Pseudoginseng Powder	Meihua
	1S	Pure Steamed Pseudoginseng Powder	Meihua
2	2R	Raw Tienchi Ginseng Tablet	Meihua
	2S	Steamed Tienchi Ginseng Tablet	Meihua
3	3R	Yunnan Tienchi Powder (Raw)	Nature's Green
	3S	Yunnan Tienchi Powder (Steamed)	Nature's Green
4	4R	Yunnan Tienchi Tablets (Raw)	Nature's Green
	4S	Yunnan Tienchi Tablets (Steamed)	Nature's Green
5	5R	Tienchi Powder (Raw)	Yunfeng
	5S	Tienchi Powder (Steamed)	Yunfeng
6	6R	Tienchi Tablets (Raw)	Yunfeng
	6S	Tienchi Tablets (Steamed)	Yunfeng
7	7R	Tienchi Powder (Raw)	Camellia
	7S	Tienchi Powder (Steamed)	Camellia
8	8R	Tienchi Tablets (Raw)	Camellia
	8S	Tienchi Tablets (Steamed)	Camellia
9	9R	Tienchi Tablet (Raw)	Yulin
	9S	Tienchi Tablet (Steamed)	Yulin
10	10R	Yunnan Tian Qi Powder (Raw)	Kiat Ling
	10S	Yunnan Tian Qi Powder (Steamed)	Kiat Ling
11	11R	Chinese Yunnan Tien Chi Tablet (Raw)	Luen Shing
	11S	Chinese Yunnan Tien Chi Tablet (Steamed)	Luen Shing

(A)

(B)

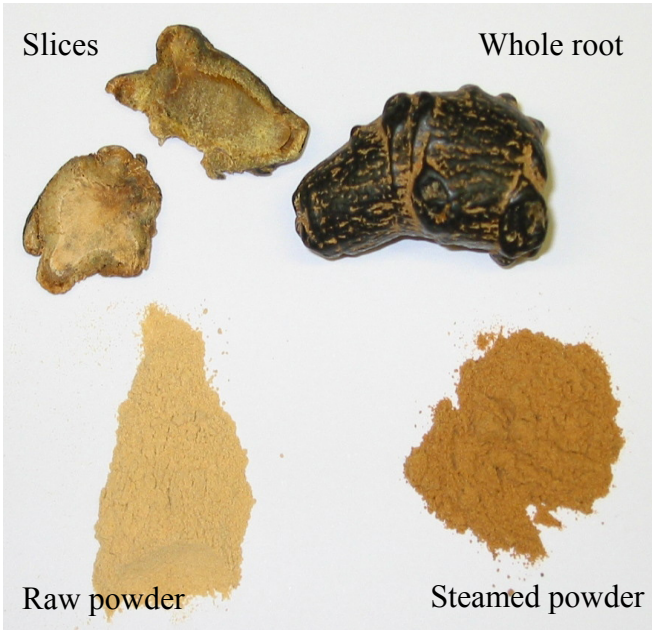


Figure 3.1. Photographs showing (A) different types of *Panax* species and (B) different forms of *P. notoginseng*.

3.1.2.2 Sample preparation

The roots were ground into powder using a laboratory blender (Waring, Connecticut, USA). 10 ml of 70% v/v aqueous methanol was added to 1 g of the powdered sample. The suspension was ultrasonically (230 V, Branson model 5510, Danbury, CT, USA) extracted for 20 min and filtered. This extraction was repeated two additional times. The combined filtrate was evaporated to dryness *in vacuo* at 40°C, using a rotary evaporator. The residue was then dissolved in 5 ml of 70% v/v methanol and filtered through a 0.45 μm nylon filter membrane prior to HPLC analysis. The solutions were then diluted for quantification.

For comparison of solvents, CPM sample pair 1 (1R and 1S) and raw *P. notoginseng* roots sample 2 were extracted with different compositions of aqueous methanol using the ultrasonication method described above. For comparison of extraction methods, CPM sample pair 1 (1R and 1S) and raw *P. notoginseng* roots sample 2 were extracted using the soxhlet method. 1 g of powdered samples were placed in the cellulose extraction thimbles and extracted with 70 ml of 70% v/v methanol for 6 h. The solvent was removed *in vacuo* at 40°C and the residue dissolved in 5 ml of 70% v/v methanol. Prior to HPLC analysis, the solutions were filtered through a 0.45 μm filter membrane.

3.1.2.3 Steaming of samples

Samples of the powdered raw *P. notoginseng* roots (Sample 2) were steamed at 105°C and 120°C using an autoclave (Hirayama, Japan) for various durations (1, 2, 3, 6 and 9 h). The powder was then dried in a vacuum oven at about 80 °C until constant weight and extracted using ultrasonication as described above.

For comparisons with steamed *P. notoginseng*, a powdered *P. ginseng* root was also steamed at 120°C using autoclave for various durations (3, 6 and 9 h), dried and extracted using similar methods.

3.1.2.4 Standards preparation

Ginsenosides Rf (0.5 mg/ml), Rb2 (0.5 mg/ml) and Rb3 (0.5 mg/ml) standards were prepared in 70% methanol. Three composite stock solutions were prepared in 70% v/v methanol for the calibration curves of notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc and Rd. Composite solution 1 consisted of notoginsenoside R1 (0.9 mg/ml) and ginsenoside Re (0.6 mg/ml). Composite solution 2 consisted of ginsenosides Rg1 (0.5 mg/ml) and Rb1 (0.5 mg/ml). Composite solution 3 consisted of ginsenosides Rc (0.6 mg/ml) and ginsenoside Rd (0.5 mg/ml). The 3 composite stock solutions were diluted with 70% methanol to give 5 to 6 different concentrations for the calibration curves. Each concentration was analysed in triplicate.

3.1.2.5 HPLC method for qualitative and quantitative analysis

An Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) equipped with quaternary gradient pump, autosampler and diode array detector (DAD) was used. A HPLC method was developed using a reversed phase column (Waters Symmetry C18, 250 x 4.6 mm I.D., 5 μm). The binary gradient elution system consisted of water (A) and acetonitrile (B) and separation was achieved using the following gradient: 0-30 min, 20% B; 30-60 min, 20-45% B; 60-78 min, 45-75% B; 78-80 min, 75-100% B. The column temperature was kept constant at 35°C. The flow rate was 1 ml/min and the injection volume was 5 μl. The UV detection wavelength was set at 203 nm and

DAD was scanned from 190 to 400 nm. HPLC chromatograms and data were acquired and processed using Agilent ChemStation software.

The concentrations of 6 saponins (notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc and Rd) present in all the samples were determined and compared.

3.1.2.6 Method validation

The calibration curves were analysed using linear regression model (least squares method). The correlation coefficients were determined. The calibration curves, residuals and standardised residuals were visually inspected to assess linearity.

The instrument/ injection precision (repeatability) was obtained by analysing the peak area variations of 13 injections of each saponin standard. The intra- and interday repeatability of the method was evaluated using multiple preparations of a sample. 5 replicate samples (raw *P. notoginseng* sample 2) were extracted and analysed in a single day and on 3 different days, i.e, a total of 15 replicated extractions of the sample were obtained. Calibration curves were generated each day before the analysis of samples. The intra- and interday variations (in relative standard deviations, RSD) of the concentrations of six saponins within the samples were calculated. The results were analysed by one-way analysis of variance (ANOVA) to determine any significant difference between the days.

The recoveries of the saponins were determined by the standard addition method. The sample (raw *P. notoginseng* sample 2) was spiked with notoginsenoside R1 (0.7 mg), ginsenosides Rg1 (1.2 mg), Re (0.9 mg), Rb1 (1.2 mg), Rc (0.5 mg), and Rd (0.6 mg) and extracted as described above. For comparison, an unspiked sample was concurrently prepared in similar way and analysed.

3.1.2.7 LC-MS

LC-MS was carried out for unambiguous and confirmatory identification of the peaks. A Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA) coupled to a ThermoQuest TSP P4000 quaternary pump liquid chromatography system with autosampler (TSP AS3000) was used. Separation was carried out using Phenomenex Luna C18(2) microbore column (150 x 2 mm id, 5 μm). The binary gradient elution system consisted of 10 mM ammonium acetate in water (A) and acetonitrile (B). The following gradient was used: 0-15 min, 20% B; 15-40 min, 20-40% B; 40-70 min, 40-75% B; 70-72 min, 75-100% B. The flow rate was 0.2 ml/min and the injection volume was 5 μl. The MS conditions were optimised. An electrospray ionisation (ESI) interface with positive-ion mode was employed. The ESI conditions were as follows: source voltage 4.5 kV, capillary temperature 230°C, sheath gas flow 80 units, auxillary gas flow 20 units, capillary voltage 10 V. Chromatograms and data were acquired and processed using Finnigan Xcalibur software (San Jose, CA, USA).

3.1.2.8 Data analysis and hierarchical clustering analysis

Statistical data analyses were performed using unpaired Student's *t*-test (for comparisons between two groups) and one-way analysis of variance (ANOVA) (for comparisons between three or more groups). Data were considered to be statistically significant when p values were <0.05. Hierarchical clustering analysis (HCA) was performed using SPSS (SPSS for windows 14.0, SPSS Inc., USA). Average linkage between groups was applied and Pearson correlation was selected as the measurement for HCA [Zhao *et al*., 2003].

3.1.3 Results and Discussion

3.1.3.1 Method development

Extraction is the first important step to obtain the highest yield of chemical components from the plant materials. The choice of solvents and methods are some of the parameters which will affect extraction yields. Methanol and aqueous methanol [Court *et al*., 1996; Samukawa *et al*., 1995; Li *et al*., 1996] were commonly used for the extraction of ginsenosides in studies on *P. ginseng* or *P. quinquefolium.* A range of composition, ranging from 30 to 100% v/v methanol, was initially tested. The amount of saponins extracted from samples 1R, 1S and raw *P. notoginseng* sample 2 using methanol and 70% v/v aqueous methanol was fully compared. Generally, it was found that the concentrations of saponins extracted with 70% v/v methanol were 4.1-30.6% (n=3) higher than the saponin concentration extracted with methanol (Table 3.2). The difference was statistically significant for some saponins. Therefore, 70% v/v methanol was the preferred solvent for the extraction and quantification of saponins.

Traditional extraction methods usually use heat and agitation to increase solubility of the phytochemicals and the rate of mass transfer. Soxhlet extraction has often been regarded as the standard conventional method for herbal extraction. However, it requires long extraction time of 6-12 h and the heat may degrade sensitive components. Besides soxhlet, the use of high intensity ultrasound to extract various phytochemicals from various parts of plants has been reported [Wu *et al*., 2001]. Its mechanism is mainly by the mechanical effects of acoustic cavitation, which enhances both solvent penetration into the plant materials and the intracellular product release by disrupting the cell walls [Wu *et al*., 2001]. It reduces the extraction time and prevents thermal degradation of components. Preliminary study optimised

Table 3.2. Comparison of saponins concentration (% w/w) obtained using methanol and 70% methanol as the extraction solvents (n=3)

Saponins	Extraction solvents	Concentration of saponins (% w/w, mean ± SD)		
		Sample 1R	Sample 1S	Raw *P. notoginseng* (sample 2)
R1	70% Methanol	0.786 ± 0.038	0.364 ± 0.012	0.547 ± 0.012
	Methanol	0.737 ± 0.011	0.335 ± 0.012	0.573 ± 0.009
Rg1	70% Methanol	2.411 ± 0.102	1.413 ± 0.040	1.987 ± 0.166
	Methanol	2.213 ± 0.106	1.261 ± 0.052	2.104 ± 0.042
Re	70% Methanol	0.452 ± 0.025	0.271 ± 0.013	0.387 ± 0.007
	Methanol	0.392 ± 0.024	0.229 ± 0.004	0.348 ± 0.008
Rb1	70% Methanol	2.300 ± 0.074	1.412 ± 0.094	2.629 ± 0.198
	Methanol	2.154 ± 0.066	1.159 ± 0.036	2.013 ± 0.043
Rc	70% Methanol	0.170 ± 0.010	0.176 ± 0.004	0.118 ± 0.010
	Methanol	0.143 ± 0.011	0.178 ± 0.010	0.098 ± 0.004
Rd	70% Methanol	0.617 ± 0.043	0.347 ± 0.008	0.557 ± 0.008
	Methanol	0.593 ± 0.006	0.308 ± 0.009	0.484 ± 0.011

the amount of samples to use for extraction, as the solvents may be saturated when the quantities of sample were too high [Anderson and Burney, 1998]. Three ultrasonic extractions (20 min each) were found to be sufficient to completely extract the saponins of interest. The concentrations of saponins extracted using ultrasonication and soxhlet method were compared and the results are presented in Table 3.3. For notoginsenoside R1, ginsenosides Rg1, Re, Rb1 and Rd, it was found that ultrasonic extraction gave either comparable or slightly higher quantities (n=3). The use of heat in soxhlet extraction may have slightly reduced the concentration of saponins [Court *et al*., 1996]. Furthermore, high temperatures may affect the raw samples and complicate the comparison of raw and steamed *P. notoginseng*. Ultrasonic extraction, which has the advantage of being fast and efficient at ambient temperatures as compared to soxhlet extraction, was therefore employed in this study.

The chromatographic conditions were developed using the standards, as well as a pair of raw and steamed *P. notoginseng*. Several potassium phosphate buffer-acetonitrile and water-acetonitrile gradient systems were evaluated as mobile phases. Both systems gave similar profiles for *P. notoginseng*. Water-acetonitrile system was chosen, as it was a simple system that gave sufficient separation of the saponins from the other neighbouring peaks for their subsequent quantification. This gradient program was also able to elute and give good separation of the numerous less polar components found to be present in steamed *P. notoginseng*. Co-elution of ginsenoside Rg1 and Re is a common problem reported in other studies [Lee *et al*., 2002; Soldati *et al*., 1980], especially when one component is in much higher concentration than the other. However, in this study, a relatively good baseline resolution of the two ginsenosides was achieved for quantification. A gradient program with an initial isocratic flow of 80% water for at least 30 min enabled their separation.

Table 3.3. Comparison of saponins concentration (% w/w) obtained using ultrasonic and soxhlet extraction (n=3)

Saponins		Concentration of saponins (% w/w, mean ± SD)		
	Extraction methods	Sample 1R	Sample 1S	Raw *P. notoginseng* (sample 2)
R1	ultrasonication	0.786 ± 0.038	0.364 ± 0.012	0.547 ± 0.012
	soxhlet	0.708 ± 0.031	0.274 ± 0.014	0.507 ± 0.010
Rg1	ultrasonication	2.411 ± 0.102	1.413 ± 0.040	1.987 ± 0.166
	soxhlet	2.245 ± 0.086	1.049 ± 0.081	1.939 ± 0.024
Re	ultrasonication	0.452 ± 0.025	0.271 ± 0.013	0.387 ± 0.007
	soxhlet	0.407 ± 0.019	0.210 ± 0.011	0.366 ± 0.006
Rb1	ultrasonication	2.300 ± 0.074	1.412 ± 0.094	2.629 ± 0.198
	soxhlet	2.269 ± 0.120	0.919 ± 0.088	2.552 ± 0.040
Rc	ultrasonication	0.170 ± 0.010	0.176 ± 0.004	0.118 ± 0.010
	soxhlet	0.169 ± 0.006	0.252 ± 0.006	0.123 ± 0.009
Rd	ultrasonication	0.617 ± 0.043	0.347 ± 0.008	0.557 ± 0.008
	soxhlet	0.579 ± 0.036	0.246 ± 0.016	0.521 ± 0.007

3.1.3.2 Identification of saponins

Preliminary studies showed that the protopanaxatriol group (R1, Rg1, Re and Rf) of saponins were generally eluted before protopanaxadiol group (Rb1, Rc, Rb2, Rb3 and Rd). Ginsenosides Rb1, Rc, Rd, Re, Rg1 and notoginsenoside R1 were detected in all *P. notoginseng* samples. Ginsenosides Rb2, Rf and Rb3 were absent or present below the detection limits. The peaks were identified by comparing their retention times and UV spectra with those of the standards and spiking of the standards into the extract. UV spectra of the various saponins closely resembled each other. MS detection, which provides greater specificity than UV detection, was also employed for identification purposes. Despite the differences in the LC conditions for HPLC and LC-MS studies, the elution order of the six saponins under the conditions of LC-MS was found to be similar to that obtained in the HPLC elution. Identities of the six saponins were unambiguously confirmed by comparing their mass spectra with those of the standards. Under this positive ESI mode, notoginsenoside R1 has $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ molecular ions at *m/z* 932, 950 and 955 and several characteristic product ions at *m/z* 405, 423, 441, 454, 459, 621, 680, 734, 751 and 769. The characteristic molecular and product ions of the other five saponins (ginsenosides Rg1, Re, Rb1, Rc and Rd) were similar to those reported in other positive-mode LC-MS studies [Chan *et al*., 2000; Wang *et al*., 1999] on *P. ginseng* and their structures have been discussed. Prior to this work, LC-MS study on *P. notoginseng* crude extracts, however, has not been reported previously.

3.1.3.3 Method validation

The analytical method has been validated. The linearity of the calibration curves was verified by the correlation coefficients as well as by the visual inspection

of the line, residuals and standardised residuals. The linear calibration curves, concentration range, limit of detection (LOD, signal/noise=3) and limit of quantification (LOQ, signal/noise=10) of ginsenosides Rb1, Rc, Rd, Re, Rg1 and notoginsenoside R1 are given in Table 3.4. The injection precision (repeatability) for the six saponins was found to be within the range of 1.8-2.9% (n=13). The intraday (n=3) and interday precisions (RSD) (n=15) of the six saponins in the samples were 0.7-4.0% (n=5) and 1.2-2.8% (n= 15) respectively. ANOVA analysis showed no statistical difference in results for all saponins between the 3 days, implying that the whole method was precise and consistent between different days. The recoveries of all six saponins were within the range of 97-102% (n=3, RSD<6.4%), showing that the extraction methods gave sufficiently good recoveries.

Table 3.4. Linear calibration curve, concentration range, LOD and LOQ of the six saponins

Saponins	Regression equation	r^2	Linear concentration range (mg/ml)	LOD (mg/ml)	LOQ (mg/ml)
R1	y = 1426x - 3.817	0.9988	0.180-0.600	0.013	0.042
Rg1	y = 2034.5x + 2.589	0.9997	0.100-0.500	0.011	0.036
Re	y = 1407.6x - 12.471	0.9989	0.120-0.400	0.008	0.027
Rb1	y = 1429.7x + 12.209	0.9993	0.100-0.500	0.010	0.033
Rc	y = 1247.2x + 5.198	0.9997	0.030-0.300	0.009	0.029
Rd	y = 1618.7x + 7.707	0.9996	0.050-0.500	0.012	0.039

3.1.3.4 Qualitative and quantitative comparisons of different *Panax* samples

The chromatographic profiles of extracts of *Panax* species were compared to see the applicability of the optimised method in differentiating them as well as to correctly identify *P. notoginseng* for quality control purposes. Each species obtained from three or more different sources was analysed. It was found that the profiles between sources were similar qualitatively. The chromatograms of *P. notoginseng*, *P. ginseng* and *P. quinquefolium* showed some consistent differences and their typical chromatograms are shown in Figure 3.1. For *P. notoginseng*, ginsenoside Rg1 was present in higher quantities than ginsenoside Re (Rg1/Re ratio of 4.68). The opposite was obtained for *P. quinquefolium*, where the average ratio of Rg1/Re was 0.09. However, for *P. ginseng*, the difference in peak areas of ginsenoside Rg1 and Re was less (Rg1/Re was 0.53). Another important difference was the presence of notoginsenoside R1 in *P. notoginseng*. This component, however, was not detected in *P. ginseng* or *P. quinquefolium*. These differences may be used as markers to differentiate the three species. The results were consistent with studies [Chuang *et al*., 1995, Zhai *et al*., 2001] comparing the *Panax* species using different analytical methods. Another commonly reported difference between *P. ginseng* and *P. quinquefolium* was the presence of ginsenoside Rf in the former and 24(R)-pseudoginsenoside F11 in the latter [Chan *et al*., 2000]. However, both of these components were not detected in *P. notoginseng*. Quantitative analysis of the common saponins, ginsenosides Rg1, Re, Rb1, Rc and Rd, was performed for all the *Panax* samples. It was found that the average total content in *P. notoginseng* was 2.1 times higher than *P. ginseng*, and 1.5 times higher than *P. quinquefolium*. Except for ginsenoside Rc and Re, ginsenosides Rg1, Rb1 and Rd were higher in *P. notoginseng* compared to the other two species (Figure 3.2). The differences between *P.*

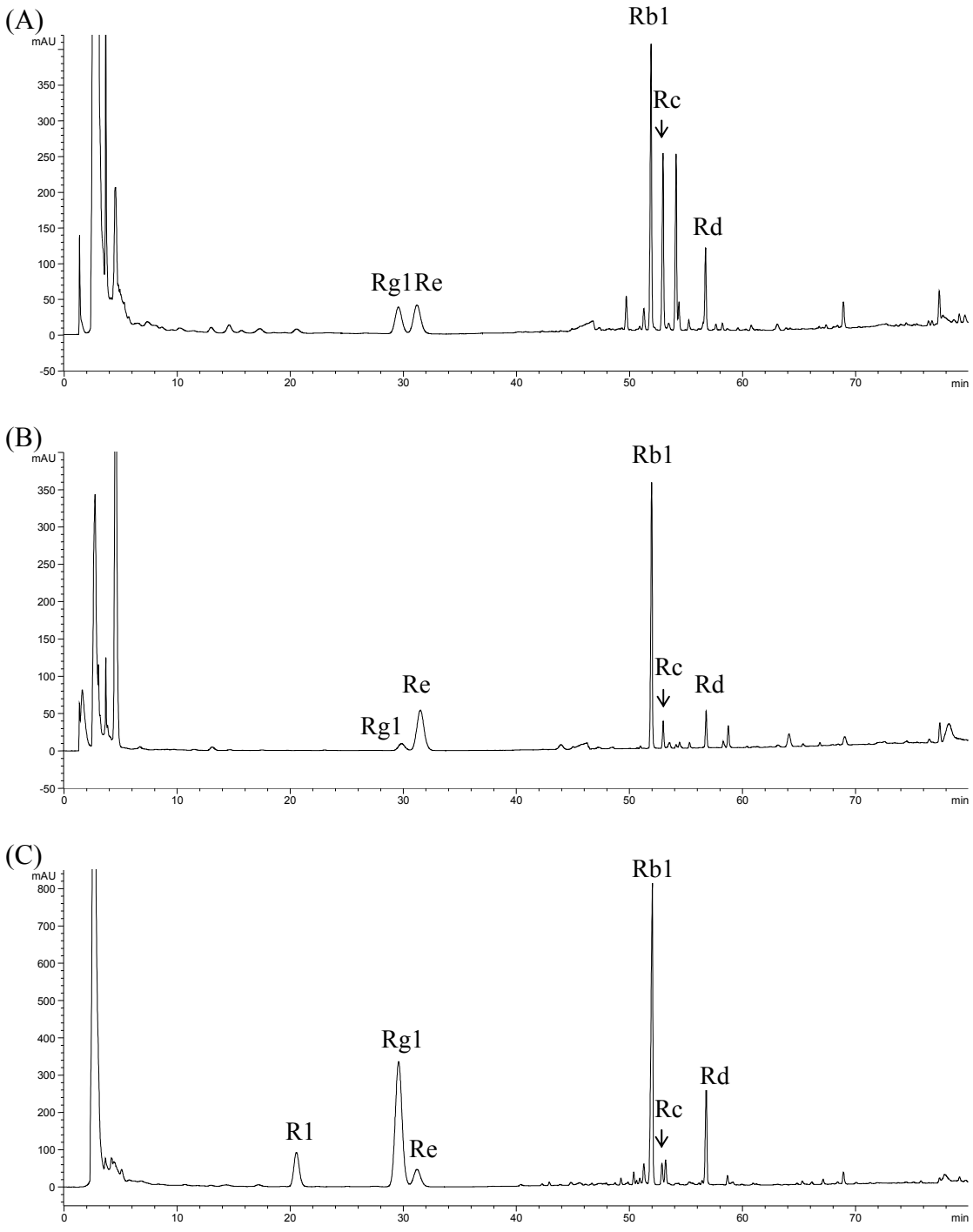


Figure 3.2. Typical HPLC chromatograms of extracts of (A) *P. ginseng*, (B) *P. quinquefolium*, and (C) *P. notoginseng*.

notoginseng and the two species were significant ($p<0.05$) for ginsenoside Rg1 and Rd. Each of these individual saponins can have differences in pharmacological activities, for example ginsenoside Rg1 and Rb1 (discussed in Section 1.4.2.4). Therefore, these differences in their relative proportions may have resulted in differences in activities of the three *Panax* species.

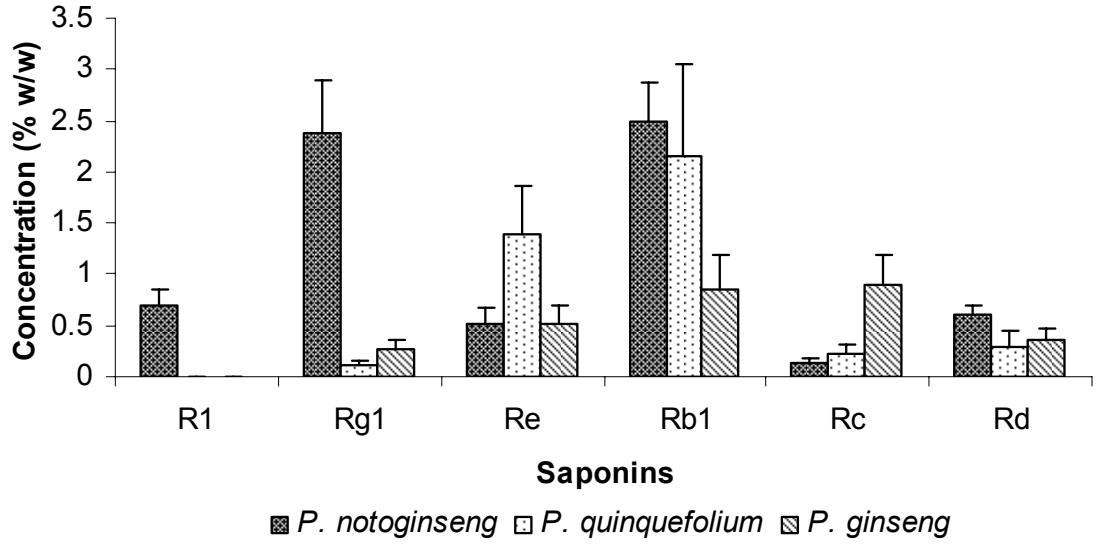


Figure 3.3. Comparison of the average concentration of saponins (% w/w) in the three *Panax* species (n>3).

Hierarchical clustering analysis (HCA) is a technique that examines the inter-point distances between all the samples and represents that information in the form of two-dimensional row spaces. To generate the dendrogram, HCA methods form clusters of samples based on their nearness in row space [Zhao *et al*., 2005]. A common approach is to initially treat every sample as a cluster and join closest clusters together. Variations of HCA use different approaches to measure distances between clusters [Zhao *et al*., 2005]. In this study, average linkage between groups was applied and Pearson correlation was used as the measurement. HCA method has the advantage of visually clustering large number of samples using many variables, instead of comparing just a few peaks. In this study, twenty five common characteristic peaks were selected and their peak areas in the fifteen samples formed a matrix of 15 x 25. The dendrogram (Figure 3.3) showed that the samples were divided into three main clusters and each cluster corresponded to each *Panax* species. From the distances, the clustering also showed that *P. ginseng* and *P. quinquefolium* were more closely related to each other than *P. notoginseng*.

Therefore, this proposed method with HCA has been shown to be able to rapidly fingerprint the three common *Panax* species and differentiate them. With this understanding of the differences between the species, this is also one step towards quality control of *P. notoginseng*, as correct authentication of the roots is important before they can be further differentiated into different forms.

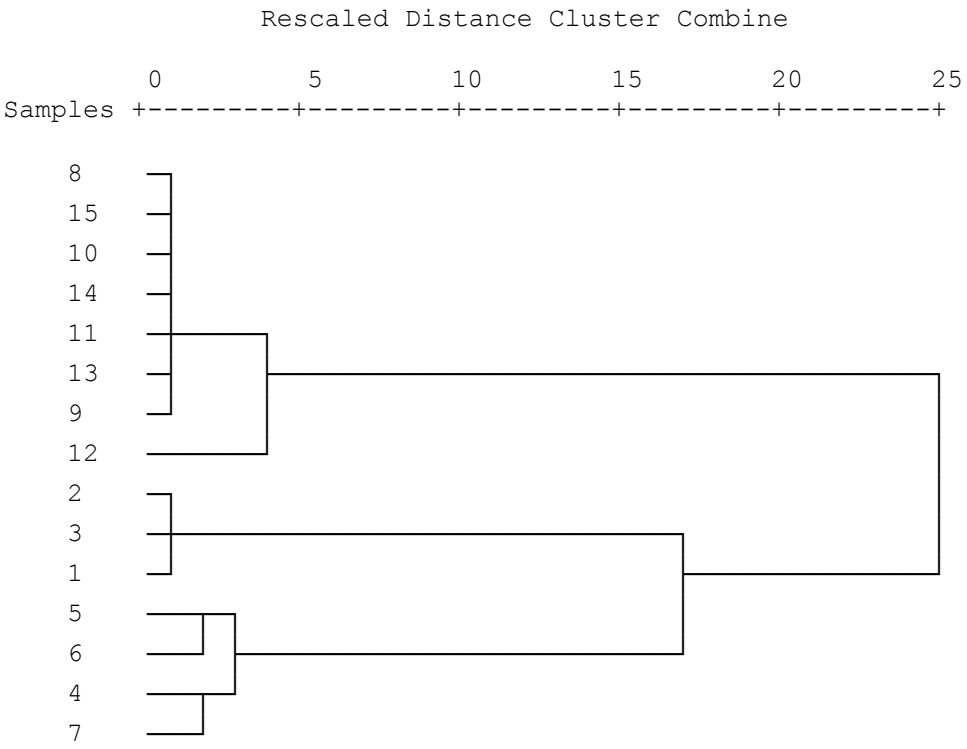


Figure 3.4. Dendrogram of the three *Panax* species using hierarchical clustering analysis with average linkage between groups. Samples 1-3 are *P. quinquefolium*, samples 4-7 are *P. ginseng*, and samples 8-15 are raw *P. notoginseng*.

3.1.3.5 Qualitative and quantitative comparisons of different raw *P. notoginseng* samples

Raw *P. notoginseng* samples obtained from different sources

When the different sources of raw *P. notoginseng* samples 1 to 8 (random, pooled samples from different sources) were compared, consistent HPLC profiles were obtained qualitatively. The major components present in all the raw *P. notoginseng* samples were ginsenosides Rg1 and Rb1. However, some variations in their concentrations were observed. The total concentrations of the six saponins were 5.1 to 9.1% w/w and the total concentrations of the four main saponins ranged from 4.6 to 8.5% w/w respectively (Table 3.5). Each of the various ratios such as R1/Rg1, Rb1/Rg1 and Rd/Rg1, were fairly consistent, without large variations or outliers. Dong *et al*. [2003] has studied a large number of samples obtained from different parts of China and found that the total concentrations of four saponins (R1, Rg1, Rb1 and Rd) ranged from 7.67 to 11.29% w/w. As the roots were obtained from different sources, some variations may be inevitable due to various factors such as strains, geographical sources, cultivation, harvest, storage and processing of the roots [Mahady *et al*., 2001]. In addition, the profiles and contents of *P. notoginseng* roots (Samples 5 and 7), which were uncoated, were found to be similar to the rest of the coated roots, showing that the coating process did not affect the saponin concentrations. When roots of three different quality or 'tou' obtained from the same source were compared, it was found that the total of the six saponins decreased with lower quality (or larger number of 'tou'). This was consistent with a study which also showed a slight decrease in four major saponins with the increasing order of the 'tou' [Dong *et al*., 2003]. Two-year-old (Y2-1 and Y2-2) and three-year-old roots (Sample

Table 3.5. Concentration of saponins (% w/w) in raw *P. notoginseng* roots obtained from different sources (n=3)

Samples	Concentration of saponins (% w/w)								Ratio of saponins		
	R1	Rg1	Re	Rb1	Rc	Rd	Total (all)	Total of 4 (R1+Rg1+Rb1+Rd)	R1/Rg1	Rb1/Rg1	Rd/Rg1
1 (1-80)	0.945	2.759	0.644	2.676	0.206	0.772	8.003	7.152	0.343	0.970	0.280
2	0.542	1.985	0.384	2.612	0.114	0.554	6.192	5.693	0.273	1.316	0.279
3	0.809	2.482	0.449	2.603	0.128	0.613	7.085	6.507	0.326	1.049	0.247
4	0.861	2.896	0.552	2.429	0.139	0.674	7.550	6.860	0.297	0.839	0.233
5	0.545	1.583	0.560	3.020	0.136	0.631	6.476	5.779	0.344	1.908	0.399
6	0.542	1.925	0.375	1.711	0.088	0.450	5.092	4.628	0.282	0.889	0.234
7	0.570	2.930	0.792	2.606	0.165	0.620	7.684	6.726	0.195	0.889	0.212
8	0.736	2.523	0.456	2.276	0.135	0.561	6.687	6.097	0.290	0.900	0.220
1-40	0.733	3.298	0.474	3.299	0.177	1.127	9.109	8.457	0.222	1.000	0.342
1-120	0.700	2.544	0.343	2.739	0.112	0.620	7.059	6.603	0.275	1.077	0.244

Sample 1-40: *P. notoginseng* sample 1, 40 ‘tou’; Sample 1-80: *P. notoginseng* sample 1, 80 ‘tou’; Sample 1-120: *P. notoginseng* sample 1, 120 ‘tou’

7) obtained from Wenshan were found to have similar profiles and contents. *P. notoginseng* injection containing PNS was analysed and found to have the same profiles as the other raw *P. notoginseng* roots except for the large peaks eluting within the first 5 min, thus showing that it has been semi-purified to contain high amount of saponins and confirming that most of the peaks seen in the chromatogram belong to the saponins class of components. Therefore, these results showed that the raw samples from different sources have similar profiles but their saponins concentrations may vary considerably depending on several factors.

Raw *P. notoginseng* individual roots obtained from the same source

Using the established and validated HPLC method, twelve individual *P. notoginseng* roots, which were randomly collected from a GAP farm in Wenshan, were studied in triplicates. Their average values are shown in Table 3.6 and the RSD of the six saponins in different extracts ranged from 1.2 to 9.6% (average of 7.1%). Except for three roots, the chromatographic fingerprints of most of the roots were consistent with the other sources, as well as that of the reference standard sample (Sample 8) (Figure 3.4). The total concentrations of the four major saponins (R1, Rg1, Rb1 and Rd) in different individual roots ranged from 4.56 to 8.91% w/w, with samples WS-3 and WS-4 having the highest total concentration of saponins while samples WS-1 and WS-2 have the lowest total concentration. The total concentrations were generally lower than the previously reported concentrations (7.67 to 11.29% w/w) in the roots obtained from different subregions of Wenshan [Dong *et al*., 2003], although the roots in this study were also obtained from similar region. For the individual saponins, notoginsenoside R1 in all the analysed samples (0.59 to 3.66% w/w) were found to be 1.8 to 15.4 times higher than the previous study [Dong *et al*.,

Table 3.6. Concentration of saponins (% w/w) (n=3) in the different individual roots obtained from the same source, compared to reference raw *P. notoginseng* root (sample 8). The values in bold were analysed to be outliers using boxplot (SPSS)

Samples	Concentration of saponins (% w/w)								Ratio of saponins		
	R1	Rg1	Re	Rb1	Rc	Rd	Total (all)	Total of 4 (R1+Rg1+Rb1+Rd)	R1/Rg1	Rb1/Rg1	Rd/Rg1
sample 8	0.736	2.523	0.456	2.276	0.135	0.561	6.687	6.097	0.29	0.90	0.22
WS-1	0.787	2.265	0.310	1.263	0.069	0.245	**4.940**	**4.560**	0.35	0.56	0.11
WS-2	1.110	1.340	0.259	1.903	0.084	0.280	**4.976**	**4.633**	0.83	1.42	0.21
WS-3	1.950	**3.820**	**0.853**	2.569	0.161	0.573	**9.928**	**8.913**	0.51	0.67	0.15
WS-4	0.623	1.483	0.152	**5.351**	0.109	**1.185**	**8.903**	**8.642**	0.42	**3.61**	**0.80**
WS-5	**3.658**	1.069	0.084	1.554	0.013	0.295	6.674	6.576	**3.42**	1.45	0.28
WS-6	1.202	2.129	0.668	2.945	0.167	0.393	7.504	6.669	0.56	1.38	0.18
WS-7	0.688	2.232	0.351	2.197	0.088	0.494	6.049	5.610	0.31	0.98	0.22
WS-8	1.527	2.242	0.310	3.419	**0.344**	0.496	8.338	7.684	0.68	1.52	0.22
WS-9	0.593	3.033	0.335	2.221	0.086	0.510	6.778	6.357	0.20	0.73	0.17
WS-10	1.338	2.623	0.644	1.759	0.091	0.368	6.823	6.088	0.51	0.67	0.14
WS-11	2.327	1.695	0.395	2.045	0.080	0.456	6.998	6.523	**1.37**	1.21	0.27
WS-12	1.010	2.448	0.176	2.805	0.087	0.599	7.124	6.862	0.41	1.15	0.24

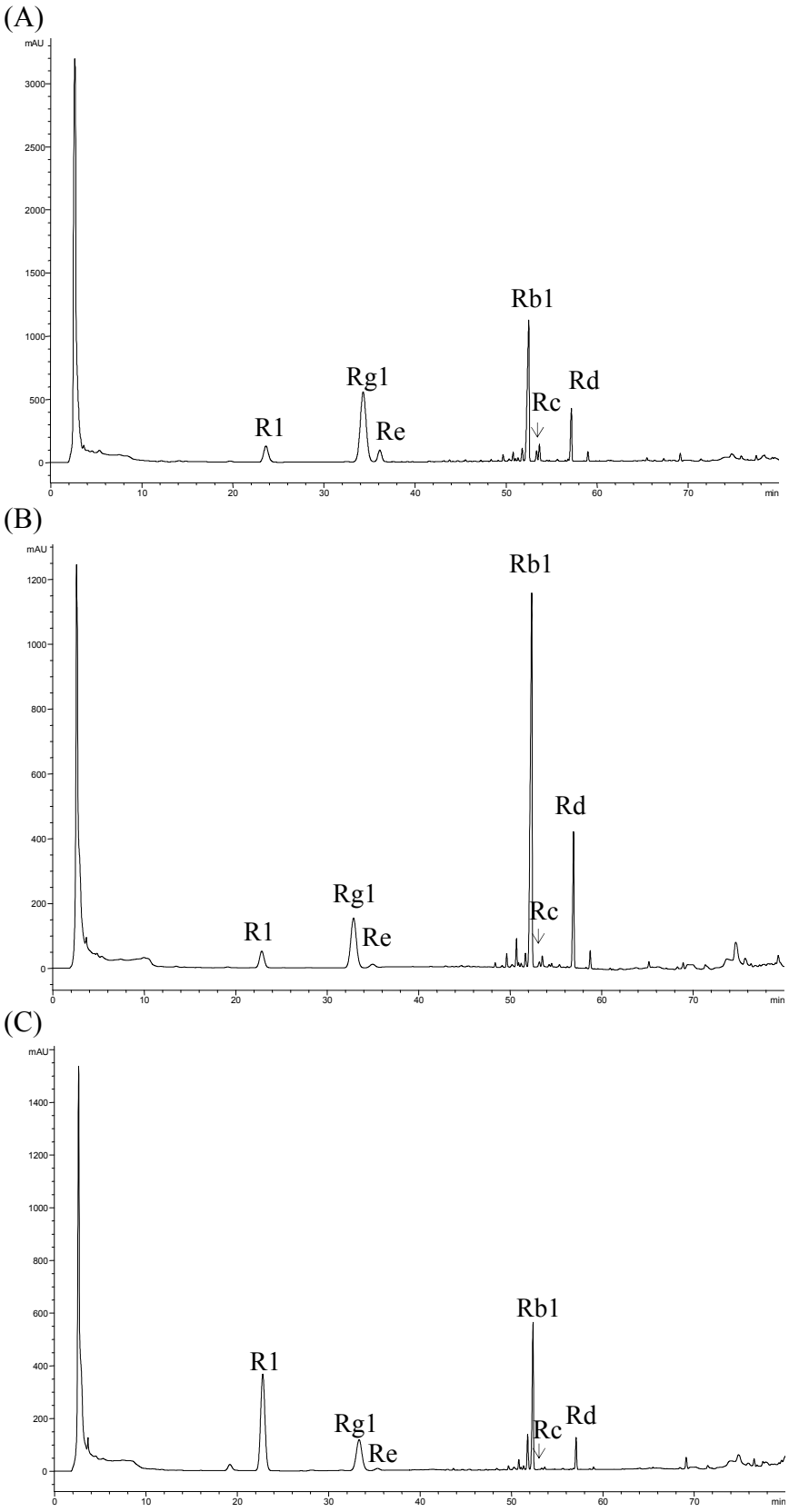


Figure 3.5. HPLC chromatograms of extracts of (A) the reference raw *P. notoginseng* sample (Sample 8), (B) sample WS-4 and (C) sample WS-5 from a GAP farm. Majority of the roots showed the typical fingerprints as in (A).

2003]. This was not unexpected as the samples may be obtained from different cultivation sites or farms and the concentrations do vary between sources (as shown by the above results in Section 3.1.3.5).

However, out of the twelve roots, there were three roots that have distinct profiles. The R1/Rg1 ratios in samples WS-5 and WS-11, and Rb1/Rg1 and Rd/Rg1 ratios in sample WS-4 were statistical outliers (analysed using Boxplots from SPSS) compared to the respective ratios in other samples (Table 3.6). Abundance of Rb1 and Rd in relative to Rg1 were increased for sample WS-4 (Figure 3.4B and Table 3.6). The Rb1/ Rg1 and Rd/Rg1 ratios were 3.61 and 0.80 in sample WS-4 compared to 0.90 and 0.22 in the reference standard sample (Sample 8) respectively. There was a substantial increase of R1 relative to Rg1 (Figure 3.4C and Table 3.6) for sample WS-5, from a ratio of 0.29 in the reference sample to a ratio of 3.42. Similarly for sample WS-11, the ratio of R1 to Rg1 was 1.4. Compared to the previous study [Dong *et al*., 2003], R1/Rg1 ratios were much higher for all the samples. Since each of these individual saponins can have differences in pharmacological activities, therefore these differences in their relative proportions may possibly affect its overall and specific activities. For example, ginsenoside Rg1 promotes angiogenesis, while ginsenoside Rb1 inhibits angiogenesis. Since these two components have opposing effects, the ratio Rb1/Rg1 has been found to be important [Sengupta *et al*., 2004]. The differences in chemical composition could be a result of differences in the genetic makeup of *P. notoginseng* plants. As the GAP farm is located in a mountainous region and covers a large area, growth conditions such as soil pH or soil moisture content, the amount of solar radiation may differ for different sections of the farm. Therefore, these subtle variations in growth conditions may have affected the chemical composition of the roots.

Hierarchical clustering analysis was used to compare the chromatograms of twelve individual roots. Twenty common characteristic peaks were selected and their peak areas in the twelve samples formed a matrix of 12 x 20. Triplicate analysis was carried out. From the dendrogram generated (Figure 3.5), the samples were divided into three main clusters: samples WS-1, 3, 7, 9 and 10 as cluster one; samples WS-2, 6, 8 and 12 as cluster two; samples WS-4, 5 and 11 as cluster three. Cluster one and two were more closely related than cluster three. Similar to above discussion on their chemical concentrations and ratios, samples WS-4, 5 and 11 (cluster three) were not closely related in the cluster. The three samples appeared to be different from each other and they were the most distinct from the rest of the samples.

Most of the root samples have profiles that were similar to the pooled samples from various sources. However, these results highlighted that some of the profiles of the roots were dissimilar and there is a surprisingly large variation in the relative abundances of the saponins in the different roots obtained from the same GAP farm or source. Therefore, the assumption that samples from the same source harvested at the same time is not necessarily true in all cases. This also highlighted the general lack of quality control and uniformity in a GAP farm. Due to such quality problems, this is one reason why reproducibility of preclinical studies and clinical trials of herbal medicines is difficult. Proper sampling is definitely important for herbal samples. Therefore, it is proposed that by analysing a number of pooled samples from different sources, the typical average profiles of *P. notoginseng* samples can be determined and using the same batches of pooled typical samples for further studies may help to reduce such inconsistencies.

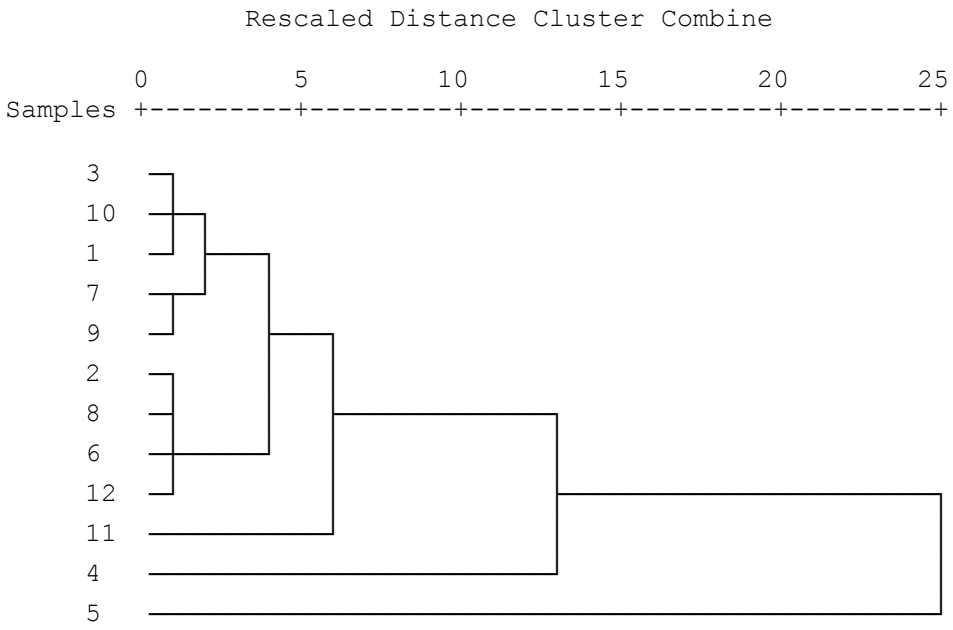


Figure 3.6. Dendrogram of *P. notoginseng* individual roots WS-1 to 12, using hierarchical clustering analysis with average linkage between groups (n=3).

3.1.3.6 Qualitative and quantitative comparisons of raw and steamed *P. notoginseng* samples

To determine the effects of steam processing on *P. notoginseng*, two representative sources of powdered raw roots were steamed. The HPLC chromatograms of raw and steamed *P. notoginseng* samples were found to be distinctively different, that is, the HPLC profiles were changed after processing (Figure 3.6). All the steamed samples from both sources showed numerous additional peaks (more than 10 peaks) eluting in the region between 63-76 min. These peaks were not distinct or were absent in the chromatograms of raw *P. notoginseng*. The numerous unique peaks, especially the four major peaks in this region (W, X, Y, Z), may serve as potential markers to differentiate the two forms.

To study the effects of different extents of steaming, a powdered raw *P. notoginseng* root was steamed at different conditions and analysed. It was found that the differences between the raw and steamed samples were more distinct when the duration of steaming was increased from 1 to 9 h (Figure 3.6). The unknown peaks were increased when the duration of steaming was increased, and more distinctive peaks (such as S to Z) may be seen after 9 h of steaming. Quantitative analysis showed that notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rd were reduced upon steaming at 120°C from 1 to 9 h (Figure 3.7). Significant differences were obtained for all the saponins when the roots were steamed for more than 2 h, at 120°C. There were no significant differences between samples that were steamed for 6 and 9 h for ginsenosides Re, Rb1 and Rc, showing that these saponins were no longer reduced beyond 6 h of steaming and the levels were becoming constant (as seen in Figure 3.7). At lower temperatures of 105°C, the decrease in saponins was lesser for the same duration. However, the qualitative profiles obtained by different steaming conditions

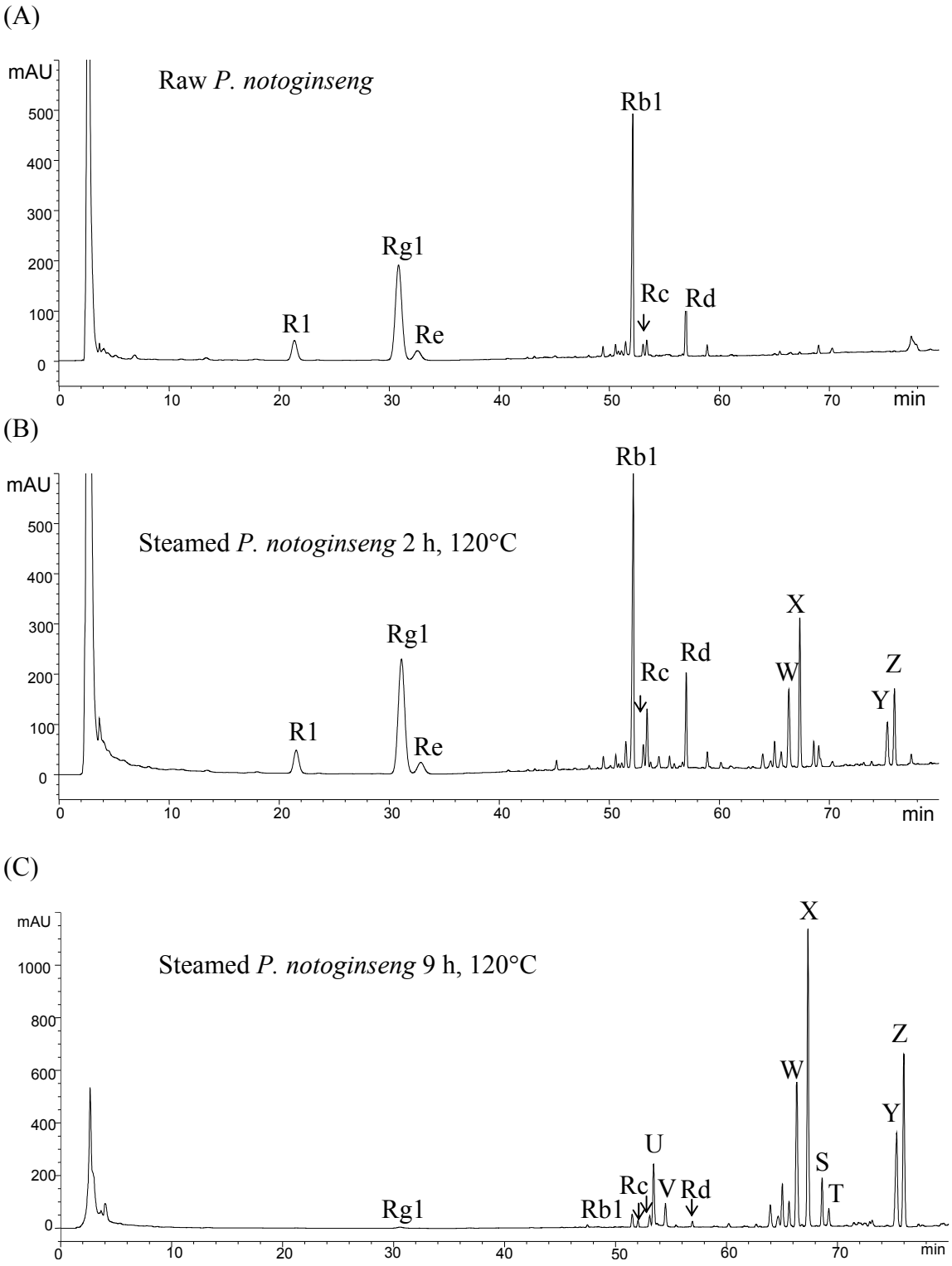


Figure 3.7. (A) Typical chromatogram of raw *P. notoginseng* (sample 2); (B) Chromatogram of steamed *P. notoginseng* (steamed for 2 h at 120°C), showing the main characteristic W, X, Y and Z peaks; (C) Chromatogram of steamed *P. notoginseng* (steamed for 9 h at 120°C), showing further increases in the characteristic S, T, U, V, W, X, Y and Z peak areas and reductions in notoginsenoside R1, ginsenosides Rg1, Re, Rb1 and Rd peak areas.

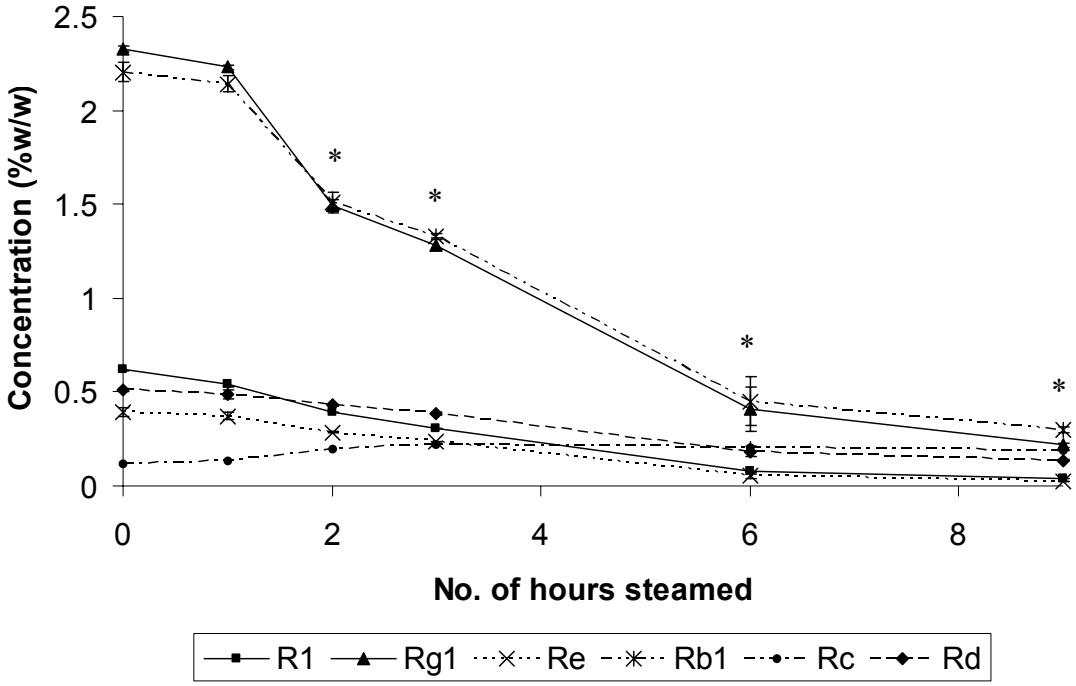


Figure 3.8. Concentrations of saponins (% w/w) in *P. notoginseng* before and after steaming for 1, 2, 3, 6 and 9 hours (n=3) (* $p<0.05$ for all the saponins, compared to the values at time 0 h, using one-way ANOVA).

and heating methods such as the use of oven for the same duration of 9 h, were similar. Autoclave method was more efficient and faster than other methods of heating the powdered roots. Furthermore, autoclaving has the advantage of providing a more accurate way of controlling the steaming temperature and duration than traditional steaming methods. It may be suitable for development into a GMP process for the steaming of traditional herbs. A greater decrease in the concentrations of the above saponins would therefore imply that the samples were steamed to a greater degree. The effect of steaming extent on *P. notoginseng* has not been previously studied in other reports. These results showed the implications and importance of standardising the steaming duration and temperature in the processing of herbal medicines.

The results in this study were consistent with a report [Yang *et al*., 1985], which isolated lower amounts of ginsenosides Rg1, Re, Rb1, Rd and notoginsenside R1 from steamed *P. notoginseng* using column chromatography. These changes in concentrations might have occurred due to chemical degradation and conversion of some thermolabile ginsenosides to other components at high temperatures during the steaming process, as were reported for *P. ginseng* and *P. quinquefolium* [Kim *et al*., 2000; Ren and Chen, 1999]. As discussed in Section 1.4.2.3, it has been postulated that the changes in saponins were due to the initial breakdown of the sugar moiety at the C-20 position, followed by further rearrangement of groups, to give rise to new saponins [Kim *et al*., 2000; Kwon *et al*., 2001; Yang *et al*., 1985]. However, except for one report [Yang *et al*., 1985], most of these changes and analysis were only reported in *P. ginseng*.

P. ginseng roots were steamed, analysed and compared to steamed *P. notoginseng*. The chromatographic profiles are shown in Figure 3.8. As the raw forms of both species were different, their steamed forms may also have distinct profiles despite the presence of some similar saponins. It was noted that the relative proportion of the unknown marker peaks were different for the two species steamed with the same conditions. For example, marker peaks W and X were higher in concentration than peaks Y and Z for *P. notoginseng*, but this trend was opposite for *P. ginseng*. Korean red ginseng and the reference standard red ginseng are examples of steamed forms of *P. ginseng* and have similar profiles to each other (Figure 3.8). From the profiles, steamed *P. ginseng* samples obtained by the autoclaving method had been steamed to a greater extent as compared to the reference red ginseng or Korean red ginseng. Quantitative analysis of ginsenosides Rg1, Re, Rb1, Rc and Rd in *P. ginseng* that were steamed 3 to 9 h (Figure 3.9) also showed similar decreasing trends as the

steamed *P. notoginseng* roots. Most of the saponin concentrations in Korean and reference red ginseng were also lower than that obtained in *P. ginseng*. Therefore, these saponins were changed upon thermal processing.

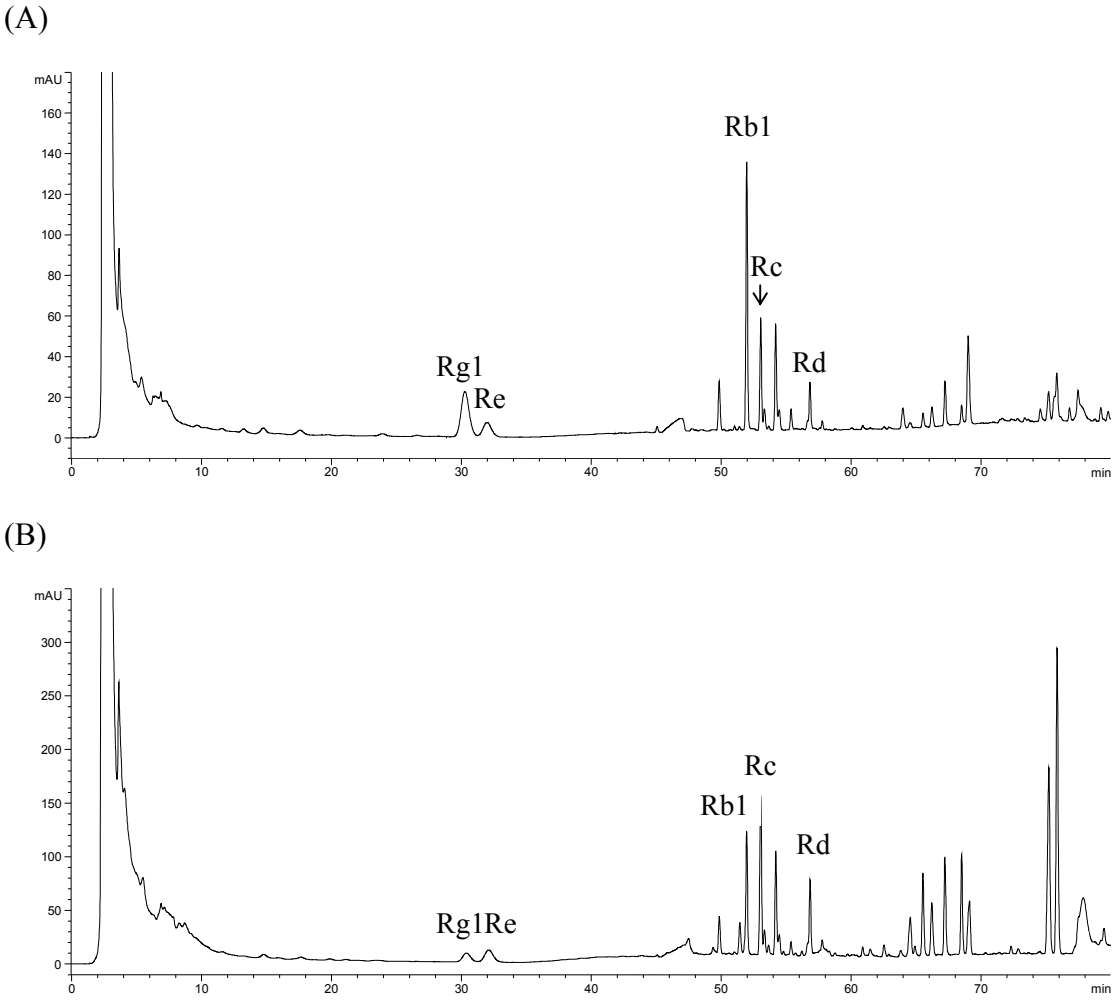


Figure 3.9. Typical chromatogram of extracts of (A) Korean red ginseng; and (B) steamed *P. ginseng* (steamed for 3 h at 120°C).

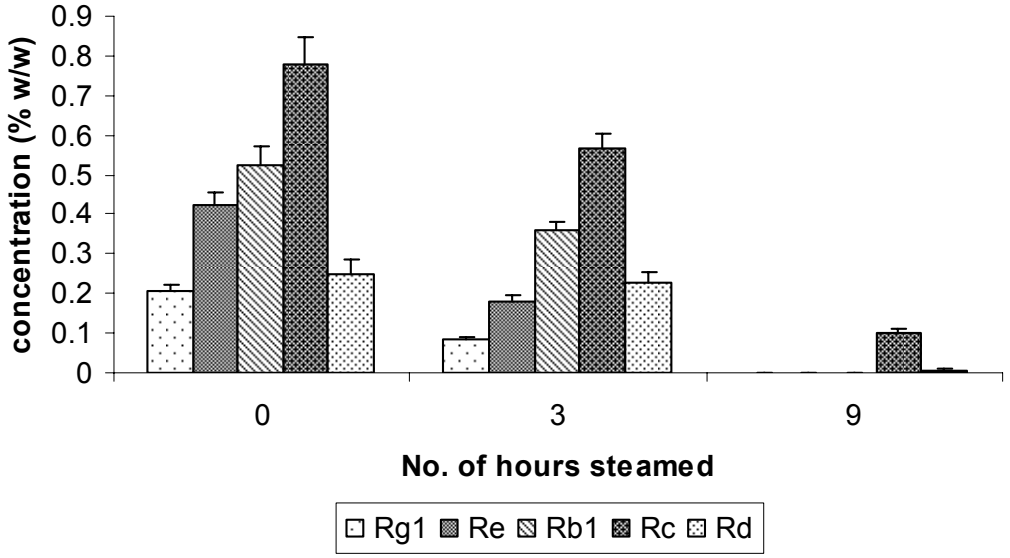
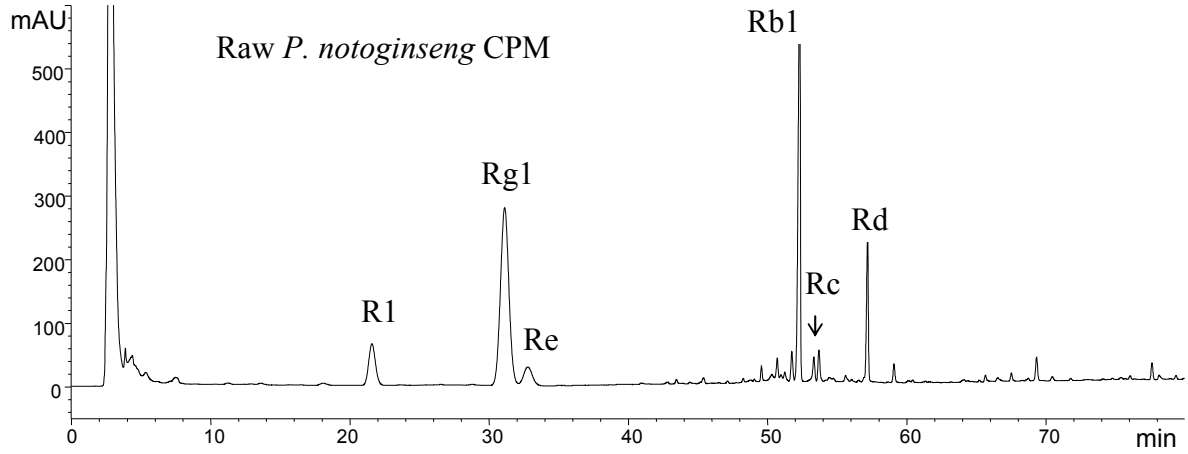


Figure 3.10. Saponins concentration (% w/w) in *P. ginseng* before and after steaming for 3 and 9 hours (n=3).

With the above knowledge on steamed *P. notoginseng* roots, the method was then applied to eleven pairs of raw and steamed *P. notoginseng* products to see if the method was applicable to detect differences in manufactured products or CPMs. By visual comparisons, out of the eleven pairs, the chromatograms of eight pairs were consistent with the profile of raw and steamed herbs, as shown in Figure 3.6. Therefore, confirming that steaming of the roots by autoclave methods gave similar chromatographic profiles as those steamed manufactured products. One raw (sample 11R) and two steamed *P. notoginseng* CPMs (samples 9S and10S), however, were found to have chromatograms that resemble their counterpart products instead (Figure 3.10). For pairs 9 and 10, the products labelled as 'steamed' were found to have chromatograms resembling a raw sample. Whereas, for pair 11, the product labelled as 'raw' have distinctive peaks in the region 63–76 min, resembling a steamed product. This may imply possible mislabelling. In the preparation of raw samples, the raw root may have been subjected to excessive high temperatures during harvesting

(A)



(B)

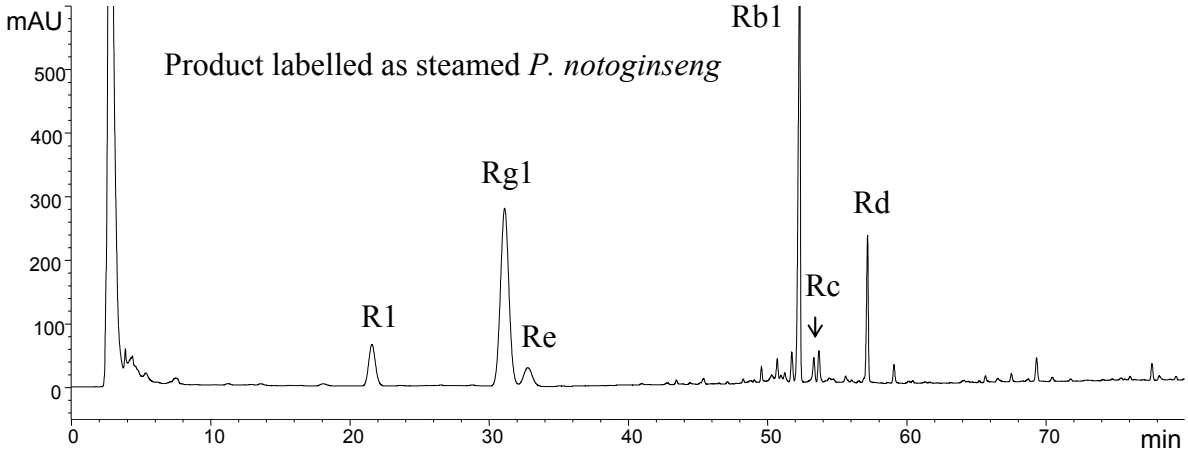


Figure 3.11. (A) HPLC chromatogram of an extract of raw *P. notoginseng* CPM (sample 10R) and (B) HPLC chromatogram of an extract of steamed *P. notoginseng* CPM (sample 10S) where the product labelled "steamed" was found to resemble a "raw" product. Note the absence of S, T, U, V, W, X, Y and Z peaks in the region between 63 –76 min in (B) despite it being labelled a steamed product.

or drying, and this may have caused some chemical components to be changed. Thus, these raw products have chromatographic profiles that resemble those of steamed products. On the other hand, if the steamed products are not steamed/ processed sufficiently, they may yield profiles similar to those of the raw products. Due to their different pharmacological indications, this may pose a danger or have different effects on patients using these products.

Quantitative analysis of the concentration (% w/w) and percentage concentration change of the saponins in all samples are presented in Table 3.7. The concentrations of ginsenosides Rg1, Re, Rb1, Rd and notoginsenoside R1 in most of the raw samples were found to be higher than those in the corresponding steamed samples (Table 3.7). Significant differences were also obtained for some pairs. The steamed CPMs may have been steamed to different degrees, resulting in great variabilities in the percentage change of saponins for the different pairs of CPMs.

The lack of quality control and standardisations of herbal products and samples are important issues to address. Currently, among the different manufacturers, to our knowledge, there is no standardisation of the processing conditions used to steam the samples. Such information is also not available or may be kept confidential by the manufacturers. As quality control and standardisation of the processing methods are currently not present, the wide variations of results for different pairs of CPMs can be expected.

Therefore, this validated method has been successfully developed and found capable of analysing various sources and products of *P. notoginseng* and its related species. Steam processing was found to change *P. notoginseng* significantly in terms of its chemical fingerprints, the compositions and concentrations of some saponins.

Table 3.7. Concentration of saponins (% w/w) (n=3) in the raw and steamed samples and the percentage change in concentration (in parenthesis) of the steamed samples (calculated with respect to the corresponding raw samples) (*p<0.05, using Student's t-test)

Pair	Samples	Concentration (% w/w) (% change)					
		R1	Rg1	Re	Rb1	Rc	Rd
1	1R	0.835	2.579	0.477	2.399	0.167	0.669
	1S	0.362 (-56.67)*	1.442 (-44.06)*	0.268 (-43.76)*	1.405 (-41.42)*	0.176 (5.49)	0.345 (-48.50)*
2	2R	0.614	2.156	0.347	2.060	0.116	0.503
	2S	0.405 (-34.10)*	1.553 (-27.96)*	0.278 (-20.00)*	1.525 (-25.98)*	0.152 (31.16)*	0.353 (-29.92)*
3	3R	0.658	2.134	0.372	2.084	0.134	0.532
	3S	0.623 (-5.35)	2.449 (14.77)*	0.442 (18.85)*	2.374 (13.91)*	0.154 (14.93)*	0.579 (8.83)*
4	4R	0.671	2.121	0.390	2.043	0.132	0.545
	4S	0.599 (-10.65)*	2.030 (-4.29)	0.373 (-4.22)	1.918 (-6.08)	0.129 (-2.35)	0.480 (-11.93)*
5	5R	0.701	2.272	0.394	2.179	0.140	0.566
	5S	0.349 (-50.27)*	1.357 (-40.26)*	0.275 (-30.11)*	1.374 (-36.93)*	0.218 (56.34)*	0.374 (-33.90)*

6	6R	0.597		1.969		0.334		1.830		0.109		0.468		
	6S	0.367	(-38.60)*	1.432	(-27.28)*	0.294	(-12.03)*	1.373	(-24.97)*	0.164	(50.88)*	0.360	(-23.09)*	
7	7R	0.596		1.874		0.321		1.739		0.107		0.483		
	7S	0.416	(-30.29)*	1.523	(-18.73)*	0.267	(-16.63)*	1.334	(-23.31)*	0.131	(22.69)*	0.377	(-21.84)*	
8	8R	0.572		1.863		0.342		1.798		0.123		0.518		
	8S	0.378	(-33.95)*	1.324	(-28.94)*	0.237	(-30.63)*	1.256	(-30.16)*	0.146	(18.75)*	0.336	(-35.15)*	
9	9R	0.624		2.149		0.352		2.137		0.132		0.490		
	9S[a]	0.620	(-0.72)	2.116	(-1.52)	0.354	(0.68)	2.092	(-2.12)	0.122	(-6.98)	0.482	(-1.57)	
10	10R	0.700		2.668		0.436		2.518		0.133		0.586		
	10S[a]	0.647	(-7.54)	2.571	(-3.64)	0.493	(13.12)*	2.370	(-5.86)	0.153	(15.29)*	0.558	(-4.75)	
11	11R[b]	0.369		1.265		0.256		1.241		0.106		0.300		
	11S	0.343	(-7.25)*	1.163	(-8.06)*	0.244	(-4.83)	1.146	(-7.71)	0.121	(14.62)*	0.285	(-5.01)	

[a]: Samples labelled as “steamed” but their chromatograms resembled that of a raw product
[b]: Sample labelled as “raw” but its chromatogram resembled that of a steamed product

3.2 Pattern matching of extracts of *P. notoginseng*

3.2.1 Introduction

Unlike synthetic drugs of high purity, medicinal herbs and their products have a very complex mixture of chemical components whose identities are only partially known. Often, a few chemical markers in the chromatograms are selected and employed in evaluating the quality and authenticity of herbs. This method does not evaluate the entire chromatographic profile and large amounts of data in the chromatograms are discarded. Furthermore, similar integration results may not be achievable if baseline resolution is not achieved in complex mixtures. Selection of suitable markers to correctly identify the herb is also difficult and subjective. Therefore, this approach is not completely sufficient for quality control of herbs. The use of chromatographic chemical fingerprinting for the identification and quality control of medicinal herbs has attracted a lot of interest [Schaneberg *et al*., 2003; Gong *et al*., 2004; Cheng *et al*., 2003; Gong *et al*., 2003]. However, due to the complex fingerprints of herbal samples and chromatographic variations, accurate analysis and interpretation of the chromatograms in chemical fingerprinting pose a great challenge to analysts.

One method to compare complex fingerprints is by visual comparison. This traditional method of visual chromatographic comparison is simple, but it is very subjective and non-quantitative. For complex chromatograms with incomplete separation of peaks, visual comparisons can be difficult and may miss subtle differences. Moreover, chromatography always varies from run-to-run due to pump, temperature, sample injection variations as well as changes in mobile phase and column chemistries. The resulting run-to-run chromatographic variations such as retention time drift and baseline drift, make the visual comparison method more

ambiguous. In some cases, these variations may make the methods analysing simple difference or variance of the chromatographic response non-applicable. Therefore, there is a need for a simple, valuable tool to objectively compare the entire chromatograms, detect real sample differences between them and measure the degree of differences quantitatively.

With the rapidly increasing computational power and rapid development of the field of chemometrics in the last two decades, it is now possible to use complex mathematical algorithms to analyse the whole chromatogram quantitatively, and automatically. Such large data sets, which are previously considered impractical, can now be handled. This approach has been applied to many types of samples including medicinal herbs [Gong *et al*., 2004; Cheng *et al*., 2003; Gong *et al*., 2003], pharmaceuticals [Welsh *et al*., 1996], and food [Cordella *et al*., 2003; Quesada Granados *et al*., 2002]. Typically, most chromatographic analysis techniques involve developing a method of identifying some peaks to be compared, developing a method of comparing the various aspects of the peaks (e.g. peak area, height), and then actually performing the comparisons. However, the inevitable variations of chromatographic peak parameters such as retention time have always been a major impediment against accurate data processing in most chemometric analysis techniques. Multivariate chemometric analysis with entire chromatographic profiles as input data, is very sensitive to even minute variations. Therefore, many new approaches for retention time adjustments/peak alignment [Gong *et al*., 2004; Johnson *et al*., 2003; Malmquist and Danielsson, 1994; Torgrip *et al*., 2003] and extracting information from the fingerprints [Cheng *et al*., 2003] have been studied to address this challenging problem for chemometric analysis. Malmquist and Danielsson's alignment algorithm [1994] involved four rounds of iterative shifting to optimise

sample-to-target correlation. Gong *et al*. [2004] used chemometrics to select marker compounds, correct the retention time shifts and reconstruct the chromatographic fingerprints with correction. Torgrip *et al*. [2003] also developed a new search algorithm, which was shown to be favourable in terms of computational speed. After alignment of chromatograms and extracting the relevant information from the fingerprints, samples can then be classified based on several multivariate data analysis methods such as principal component analysis (PCA), soft independent modelling of class analogy (SIMCA), K-nearest neighbours (KNN), linear discriminant analysis (LDA). Artificial neural networks (ANN) are also rapidly emerging in the field of analytical chemistry as a powerful tool for pattern searching, mapping, and fingerprinting. There are some studies [Welsh *et al*., 1996; Cordella *et al*., 2003] using ANN and on the comparison of ANN architectures with standard classifiers.

Besides the above chemometric analysis methods, novel chromatographic pattern matching software [Gorenstein, 1999; Waters Corporation, 2002] which combines the alignment and analysis of chromatograms will be specifically useful as a simple and convenient method for comparing chromatographic fingerprints. This method treats the entire chromatogram as a pattern and compares chromatograms in pairs. One is specified as reference (typically a known standard) against which the software compares the other sample chromatogram. In this application, the raw *P. notoginseng* samples were specified as reference. It uses a chromatographic alignment algorithm to align corresponding retention intervals from two chromatograms.

The alignment algorithm is based on a two-step procedure. First, the algorithm fixes the responses in the sample chromatogram and mathematically adjusts the reference responses by least-squares optimisation to produce the best fit between the two chromatograms. The algorithm is based on the assumption that the normal

chromatographic variations can be described by five parameters (concentration/response ratio, baseline offset, baseline drift, retention time offset, retention time scale). It measures and applies these alignment parameters to the reference chromatogram. The response ratio refers to the factor by which the reference chromatogram is multiplied along the *y*-axis to best align with the sample chromatogram. The retention time offset refers to the amount of time by which the peak apices of reference chromatogram is adjusted. Retention time scale refers to the amount by which the retention time scale (*x*-axis) of the reference chromatogram is stretched or compressed to best align with the sample. Baseline offset refers to the amount by which the reference chromatogram is adjusted in the y-axis while baseline drift refers to the slope applied to the reference chromatogram baseline. In measuring all these values, only the raw data is used and no peak integration is performed. The pattern matching algorithm simulates, or models a possible range of responses for the reference chromatogram and finds the values for the five parameters by finding those values that minimise the sum of squared differences between the two chromatograms.

Secondly, after alignment, the algorithm calculates the degree of differences (in terms of standard deviations) between the sample chromatogram and the parameter-adjusted reference chromatogram. The standard deviation is the square root of the average of a squared difference. The formula is:

$$\text{Standard deviation} = \sqrt{\frac{\sum_{i=1}^{N} (f_i - a_i)^2}{(N - 5)}}$$

where f_i are the response values in the fixed, sample chromatogram and a_i are the adjusted response values from the reference chromatogram. The index i ranges over the N time samples in the comparison interval. The value for N is reduced by 5 to take

into account the effect of the five-parameter alignment on the magnitude of differences.

During a scan of the entire chromatogram, the algorithm centers an alignment interval of fixed width (typically 2-peak widths) on each point over the entire chromatogram, regardless of the presence of peaks. It performs the alignment for each interval and computes the five alignment parameters as well as the standard deviations for each interval within the scan region (from start to stop time). From the whole scan, the pattern match standard deviation (PMSD) value is computed. It is the root-mean-square value of all the individual standard deviations measured within the entire scan region. The term "standard deviation" is used in this study and it means a value of the pairwise differences calculated by the above corresponding formula. A Matlab implementation of this alignment algorithm is available [Gorenstein, 1999].

In this method, the variations due to normal chromatographic variations will not affect the comparisons, thereby revealing only changes due to the samples. This alignment algorithm has the advantage of adjusting for five underlying chromatographic variations simultaneously. Furthermore, no selection of peaks, internal standards, or traditional peak integration is needed, compared to known techniques. The algorithm detects peaks using the second derivative of the chromatogram. The apex of the inverted second derivative identifies the apex of a peak. Besides showing how similar or different the samples are, it can also rapidly identify which specific retention time interval of the chromatograms has significant pattern differences or new peaks. This may help to identify and isolate the differentiating peaks of interest. Other than chromatograms from LC separation, it can also be applied to GC, CE separation or other imported data.

In Section 3.1, it was shown that there were visually distinct differences between the chromatograms of raw and steamed samples and the concentrations of some saponins were changed. The whole chromatograms were qualitatively compared and clustered using HCA. Therefore, this part of the study aims to use a high-performance liquid chromatographic (HPLC) pattern matching method as a new approach to objectively and quantitatively differentiate between the whole chromatograms of raw and steamed *P. notoginseng* samples [Lau *et al*., 2004]. Prior to this work, this pattern matching analysis tool has not been studied or applied to complex samples such as medicinal herbs. This method will be applied on 11 pairs of raw and steamed *P. notoginseng* CPMs to ascertain their degree of similarity or differences between each other.

3.2.2 Experimental

3.2.2.1 Materials

The water was treated with a Milli-Q water purification system (Millipore, Molsheim, France). HPLC-grade solvents were used for the analysis. The raw *P. notoginseng* roots (sample 2) and the eleven pairs of raw and steamed *P. notoginseng* CPMs were obtained from local Chinese medical shops (Table 3.1). Notoginsenoside R1, ginsenosides Rb1, Rc, Rd, Re and Rg1 standards were purchased as described in section 3.1.2.1.

3.2.2.2 Sample preparation

Sample preparation and steaming procedure were carried out using the same method as Section 3.1.2.2 and 3.1.2.3. The *P. notoginseng* samples, which were steamed at 120°C for 2, 6 and 9 h, were used. Four pairs of raw and steamed *P.*

notoginseng products, whose identities and labels were not known to the analyst, were extracted in the same way for the 'blind' test.

3.2.2.3 HPLC with chromatographic pattern matching analysis

HPLC analysis was performed on a Waters Alliance liquid chromatograph (Milford, MA, USA) equipped with Alliance separation module 2695 and photodiode array detector 2996. The reversed-phase column used was Waters Symmetry C_{18} (250 mm× 4.6 mm i.d., 5 μm). The binary gradient elution system consisted of (A) water and (B) acetonitrile. Separation was achieved using the same gradient and conditions described in section 3.1.2.5. Sample analysis was processed by Waters Empower software 2002 with chromatographic pattern matching tool.

The chromatographic pattern match processing method parameters used for the comparisons of samples were optimised as follows. Replicate injections of identical samples were carried out and used to develop method parameters, namely, scan start and stop times, peak width, alignment interval, retention time search limit, detection threshold, response value, and percent peak height. The scan start and stop times were 10 and 78 min. The detection threshold was set at 350. The response value and percent peak height were 0.0005 and 4.3%, respectively. After the parameters were optimised, the same pattern match processing method was then applied to all the samples compared.

Statistical data analysis in this study was performed using one-way ANOVA for comparisons of more than two groups. Differences were considered to be significant when p values were <0.05.

3.2.3 Results and Discussion

3.2.3.1 Optimisation of pattern match processing method

The pattern match processing method was developed with repeated (n≥6) replicate injections of the raw samples, as well as replicate injections of steamed (2 h) samples, to detect true differences between samples. The scan region of 10–78 min was chosen to include the region of interest and to exclude regions of void volume, injection artefacts at the beginning and the re-equilibration region at the end of the chromatogram. In the preliminary pattern matching method, the scan time was selected while the software automatically calculated the rest of the parameters (alignment interval, retention time search limit, peak width, detection threshold, response value, and percent peak height) for the pattern matching process. In the optimisation routine, the autocalculated values can be further optimised to suit the particular set of samples and applications of the study.

The retention time search limit affects the alignment results. It was further optimised to ensure the retention time offset encompasses the largest possible retention time offsets between the chromatograms. Retention time search limit of 15 s specified the retention time range over which the reference chromatogram was offset in search for the best alignment. The detection threshold affects the interpretation of the results. It was set to determine which peak apices were detected and plotted on the chromatogram. Detection threshold was set at 350 for optimal number of detected peaks in this case and to prevent over-clustering of peak apex markers in the plots.

To objectively determine if a difference is genuine (not due to baseline noise), the individual standard deviations should be compared against a threshold. The threshold values do not affect the alignment or the standard deviation values. It only helps in the interpretation of the results. This standard deviation threshold is obtained

from comparing similar chromatograms (replicate injections). This threshold is calculated automatically by the software from response value and percent peak height. These two parameters were further optimised to correspond to the highest standard deviation values that an interval could have when the compared samples were the same. It ensured that the standard deviations of the replicate injections (identical samples) were detected below the threshold line. Therefore, it is a kind of limit-of-detection test, which should be sensitive to detect true differences between different samples and not similar samples. The formula for the standard deviation threshold is:

$$\text{Standard deviation threshold} = \text{response} + [\text{percent peak height}/100 \times (\text{maximum peak height in the interval})]$$

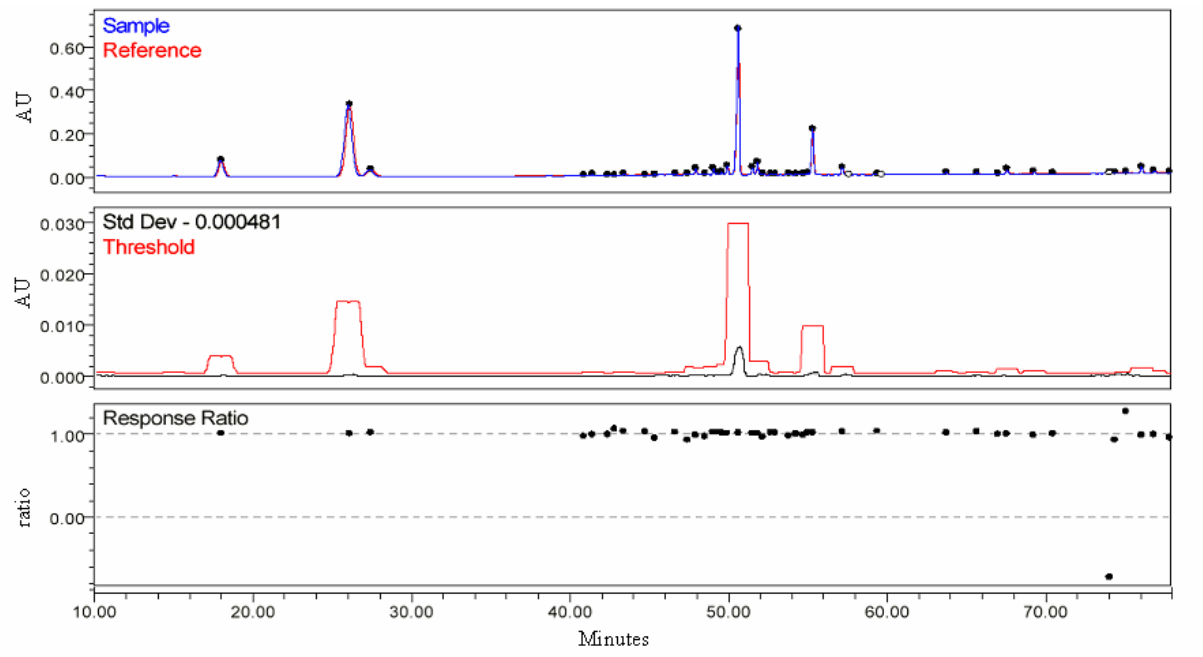
The response is the minimum value for the standard deviation threshold. The percent peak height adds a value to the response that is proportional to the peak height within the compared interval.

As described in Section 3.2.1, this tool will take into account 5 parameters of chromatographic variations to align the chromatograms, and it does not require the characterisation of peaks or other chromatographic features that are required by other known techniques. After chromatographic alignment and comparison, a quantitative value showing the relative difference between the samples will be generated and it will be used as a criterion to differentiate between raw and steamed samples in this study.

3.2.3.2 Chromatographic pattern matching results of raw and steamed *P. notoginseng*

Before comparing the raw and steamed samples, pairs (n≥6 pairs) of identical raw samples (replicate injections) were compared. Replicate injections of identical steamed samples (n≥6 pairs) were also performed and they gave similar results as replicate injections of raw samples. The typical pattern matching results of a pair of replicate injections of raw samples are shown in Figure 3.11A. The top plot shows the overlay of a pair of chromatograms in the scan region between 10 and 78 min. The middle plot shows their individual standard deviations of all points within the scan region. The standard deviation is a measure of the magnitude of point-to-point differences between the two chromatograms after alignment, as shown by the formula above. For similar samples (such as these replicate injections), the standard deviations were small and below the threshold line and they represented the background noise present. For replicate injections of raw *P. notoginseng* and replicate injections of steamed (2 h) *P. notoginseng*, the pattern match standard deviation values were found to be below 0.0006 and their average values were not statistically significant from each other (Table 3.8). Response ratios (sample/reference) of the peaks in both chromatograms were close to 1 for identical samples, as shown in the bottom plot of Figure 3.11A. The response ratios measure the approximate ratio of concentrations within the compared intervals, if the alignment is good.

(A)



(B)

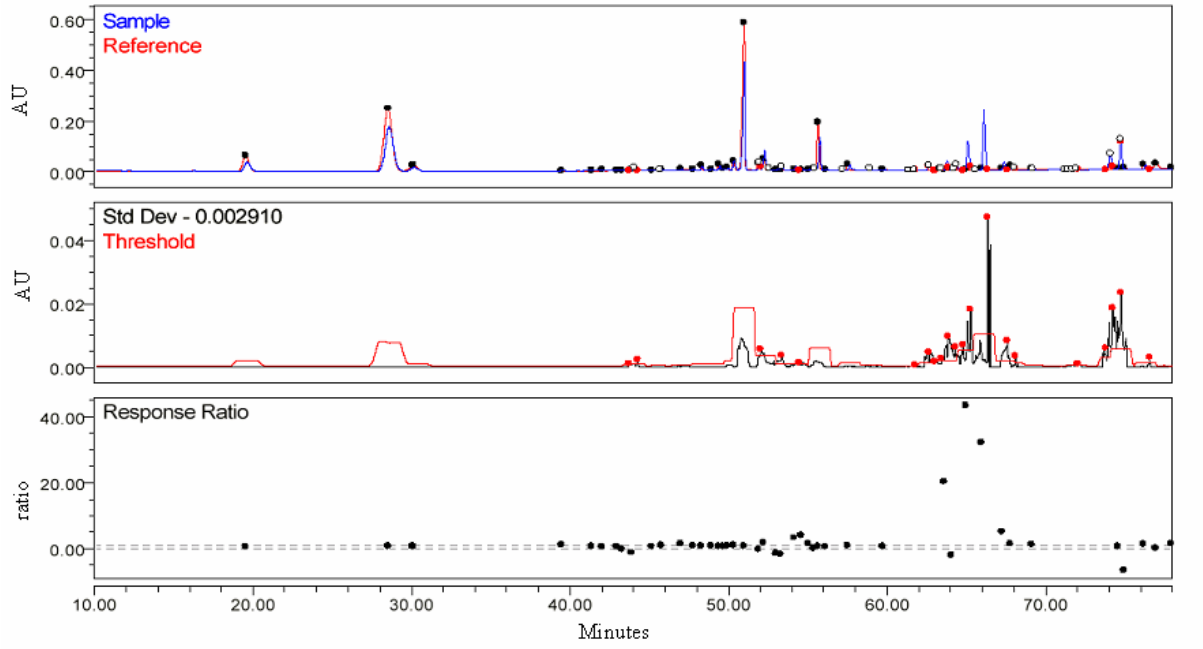


Figure 3.12. Typical results from chromatographic pattern matching for (A) replicate injections of raw *P. notoginseng* and (B) raw and steamed (2 h) *P. notoginseng*. Each of the top plots shows an overlay of the chromatograms, with black markers on peak apices. Each of the middle plots shows their corresponding standard deviations for all points in the scan region. Each of the bottom plots shows response ratios (sample/reference) of all points within scan region.

Table 3.8. Pattern match standard deviations (PMSD) of raw and steamed root samples, and the 11 pairs of CPM products (raw form is taken as the reference)

Pair no.	Samples	Brand name	PMSD (AU)
	Raw *P. notoginseng* (replicate injections)		0.0004
	Steamed *P. notoginseng* (replicate injections)		0.0004
	Steamed (2h) vs raw *P. notoginseng* sample		0.0024
	Steamed (6h) vs raw *P. notoginseng* sample		0.0096
	Steamed (9h) vs raw *P. notoginseng* sample		0.0120
1	Pure Raw Pseudoginseng Powder	Meihua	0.0041
	Pure Steamed Pseudoginseng Powder		
2	Raw Tienchi Ginseng Tablet	Meihua	0.0021
	Steamed Tienchi Ginseng Tablet		
3	Yunnan Tienchi Powder (Raw)	Nature's Green	0.0012
	Yunnan Tienchi Powder (Steamed)		
4	Yunnan Tienchi Tablets (Raw)	Nature's Green	0.0009
	Yunnan Tienchi Tablets (Steamed)		
5	Tienchi Powder (Raw)	Yunfeng	0.0050
	Tienchi Powder (Steamed)		
6	Tienchi Tablets (Raw)	Yunfeng	0.0028
	Tienchi Tablets (Steamed)		
7	Tienchi Powder (Raw)	Camellia	0.0026
	Tienchi Powder (Steamed)		
8	Tienchi Tablets (Raw)	Camellia	0.0025
	Tienchi Tablets (Steamed)		
9	Tienchi Tablet (Raw)	Yulin	0.0005
	Tienchi Tablet (Steamed)		
10	Yunnan Tian Qi Powder (Raw)	Kiat Ling	0.0004
	Yunnan Tian Qi Powder (Steamed)		
11	Chinese Yunnan Tien Chi Tablet (Raw)	Luen Shing	0.0004
	Chinese Yunnan Tien Chi Tablet (Steamed)		

Using pattern matching analysis, the raw and steamed samples were successfully differentiated. The top plot of Figure 3.11B shows the typical overlaid chromatograms of raw and steamed (2 h) *P. notoginseng*. The standard deviations increased above threshold line at various points in the scan region, especially in the region 63–76 min, as shown in the middle plot of Figure 3.11B. This indicated that the samples were not similar in this region. Response ratio also increased to a value of 45 at one point (65^{th} minute). The pattern match standard deviation value was also significantly higher than that obtained from replicate injections of samples. As the duration of steaming increased, the differences between the chromatograms also increased. This was reflected in the pattern match plot. The average pattern match standard deviation values increased with duration of steaming (Figure 3.12). The values (Table 3.8) increased from 0.0024 to 0.0120 for 2 and 9 h of steaming, respectively. These were about 3 to 17 times the PMSD values for replicate injections of identical samples and the PMSD values were statistically higher ($p < 0.05$) than that obtained for replicate injections. The values obtained for steamed samples (2, 6 and 9 h) were also statistically significant from each other. Therefore, these values gave useful indications of the similarity of the sample pairs and served as a similarity or match index.

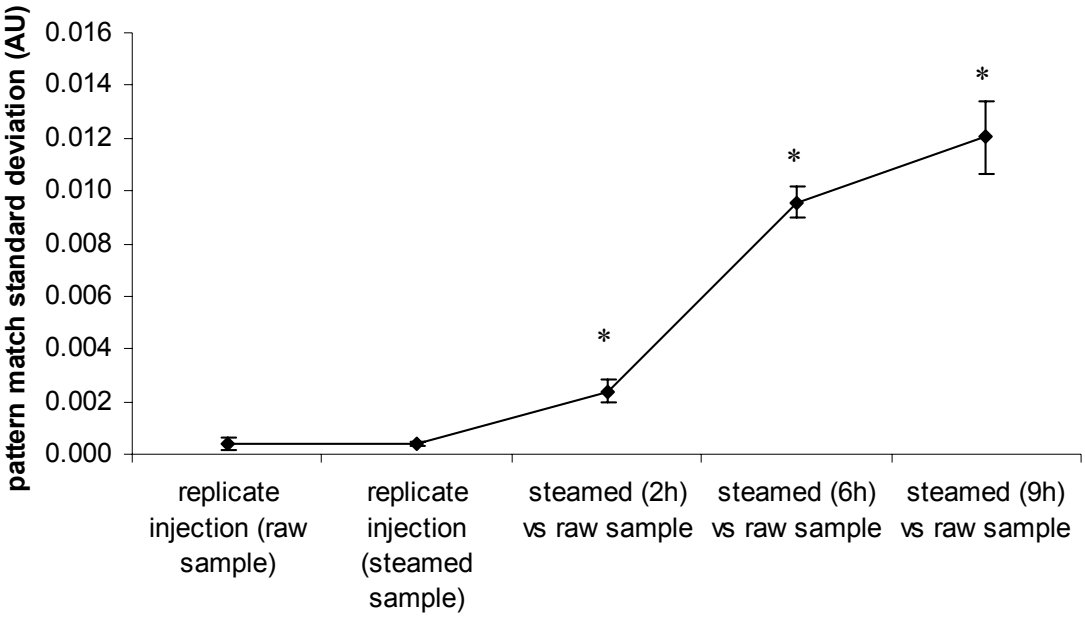


Figure 3.13. Pattern match standard deviation values of replicate injections and *P. notoginseng* samples that were steamed for 2, 6 and 9 h. Values were means ± SD, n≥6. For the steamed samples, the pattern match standard deviation values were obtained from the pattern matching comparisons with the corresponding raw sample (before steaming). The asterisk (*) denotes statistically significant differences between the PMSD values of the steamed samples and replicate injections at $p<0.05$.

3.2.3.3 Chromatographic pattern matching of raw and steamed *P. notoginseng* products

After establishing the method and differentiating the known raw and steamed samples, the chromatographic pattern matching method was then applied to the comparisons of 11 pairs of raw and steamed *P. notoginseng* proprietary herbal products. Their pattern match standard deviation values are summarised in Table 3.8. Eight pairs (pairs 1–8) of raw and steamed CPMs showed distinctive differences from the pattern match plots. The pattern match standard deviation values of these eight pairs ranged from 0.0009 to 0.0050. Values above 0.0008 were found to be statistically different ($p < 0.05$) from the replicate injections of identical samples. Similar to the above study (Section 3.1), three raw and steamed pairs (pairs 9–11) were found to have similar chromatographic patterns from their pattern match plots. The pattern match standard deviation values for the three pairs were close to that obtained for replicate injections. The standard deviations were below threshold line and their response ratios were close to 1, which were similar to the typical results of the replicate injections. This implies that these pairs of sample were similar, although they were labelled as 'raw' and 'steamed'. Although the above study (Section 3.1) reported the clear differentiation of chromatographic fingerprints of extracts of raw and steamed *P. notoginseng,* the current method provided further quantitative comparisons of pairs of such products. Visual chromatographic comparisons of the extracts of three pairs out of eleven pairs of such products showed that the differentiation between the pairs was not clear and was inconsistent with the label claims. Indeed, upon quantitative comparisons of the chromatograms, we have demonstrated that the method is able to detect such discrepancies.

From the values of the pattern match standard deviation, additional useful information regarding the degree in which the raw herb was steamed or changed by the process may be obtained. This information may not be objectively obtained by visual comparisons or clustering methods. Among the CPMs, pair 10 has the lowest PMSD value, while pair 5 has the highest value. The greater the differences, the greater the degree in which the herbs were steamed or the components were changed by the processing method, with respect to the corresponding raw sample. This is useful in standardisation of the steamed herb whereby a particular target range may be set. A 'blind' test was carried out whereby the identities of four pairs of raw and steamed products were not known to the analyst. Using pattern matching analysis, accurate identification of the raw and steamed products was obtained, confirming the usefulness of this method.

Therefore, this method provided quantitative differentiation of raw and steamed *P. notoginseng* samples and was capable of showing the degree in which steaming has changed the samples. It may be applied to identify herbs, check the quality or source by comparing an unknown with a known or standard herb. Using the pattern match standard deviation values, it can determine which samples are most similar to the given reference and provide the closest match.

3.3 Conclusion

In this chapter, a new, simple and reliable HPLC-DAD analytical method has been successfully developed and found to be suitable for the routine differentiation between raw and steamed forms of *P. notoginseng*. The method has been validated for linearity, precision, specificity, LOD and LOQ, and can be used for the simultaneous quantification of six saponins present in both forms. Together with HCA, it was applicable for the authentication of raw *P. notoginseng* from its closely related *Panax* species. Raw *P. notoginseng* samples from different and same sources were also investigated. This is the first report of qualitative and quantitative comparisons of the saponins concentrations in both raw and steamed *P. notoginseng* using HPLC-DAD. The concentrations were decreased upon steaming and several new unknown peaks were increased. The developed method has been successfully applied to 11 pairs of raw and steamed *P. notoginseng* CPMs. Three products were found to show discrepancies between their labelled claims (raw or steamed) and the results of analysis.

In addition, a new HPLC method coupled with chromatographic pattern matching analysis further enhanced the method by allowing rapid, simple, automated, and quantitative comparisons of complex chromatograms. The method was successful in providing quantitative differentiation of raw and steamed *P. notoginseng* root samples and its products, and in rapidly detecting inconsistencies in the products. The degree of similarity and degree in which the raw sample has been changed by the steaming process can be deduced. Therefore, these methods have important applications and they are proposed as potentially useful tools for ensuring quality and safety of herbal medicines.

CHAPTER 4

ISOLATION AND IDENTIFICATION OF CHEMICAL COMPONENTS FROM STEAMED *PANAX NOTOGINSENG*

4.1 Introduction

The chemical components from *Panax* species have been studied and the largest class of bioactive components is found to be saponins. More than 30 ginsenosides have been isolated and structurally elucidated, but there is still ongoing research on the components of *P. ginseng*, including new and minor components. The components obtained from different species and different parts of the same plant may be different. The types of saponins that are present in raw *P. notoginseng* roots have been reported [State Administration of Traditional Chinese Medicine, 1996; Zhou *et al*., 1981; Taniyasu *et al*., 1982; Matsura *et al*., 1983; Zhao *et al*., 1996; Yoshikawa *et al*., 1997a; Yoshikawa *et al*., 1997b; Yoshikawa *et al*., 2001; Ma *et al*., 1999; Yang *et al*., 1983]. However, to date, the chemical components in steamed *P. notogoinseng* have not been as well-studied as the raw form. Prior to this work, only one study [Yang *et al*., 1985] was found (Section 1.4.2.3). However, there may be more components in the steamed form than the previously reported saponins. Subsequent studies are needed to further understand the differences between them. As discussed in Chapter 3, the chromatograms of raw and steamed *P. notoginseng* were different and the steamed form contained many additional peaks with unknown identities. These components are important in differentiating the two forms and may result in different activities. Although there were some chemical studies on red or steamed ginseng [Kim *et al*., 2000; Kwon *et al*., 2001; Ren and Chen, 1999; Kitagawa *et al*.,

1987; Kasai *et al*, 1983; Ryu *et al*, 1997; Baek *et al*., 1996; Park *et al*., 1998], the results may not be directly extrapolated to *P. notoginseng*.

Therefore, the objective of this study is to isolate and identify the main biomarkers in steamed *P. notoginseng*, which can serve to differentiate the raw and steamed forms, to better explain their differences and to understand the main components that are present in the steamed form [Lau *et al*., 2004].

4.2 Experimental

4.2.1 Materials

The raw *P. notoginseng* roots (sample 2), notoginsenoside R1, ginsenosides Rb1, Rc, Rd, Re and Rg1 standards were purchased (Section 3.1.2.1). 20R- and 20S-ginsenoside Rg3 were generous gifts from Professor J.H. Park (College of Pharmacy, Seoul National University, Korea). 20RS-ginsenoside Rg2 mixture and 20S-ginsenoside Rh2 were obtained from Delta Information Centre for Natural Organic Compounds, China.

The solvents used for extraction and separation were of analytical grades, while the solvents used for HPLC were of HPLC-grade. The water was treated with Milli-Q water purification system (Millipore, Molsheim, France). Silica gel 60 (63-200 μm, 70-230 mesh) for column chromatography, and TLC aluminium sheets pre-coated with silica gel 60 (layer thickness 0.2 mm, 20 x 20 cm) were purchased from Merck KGaA (Darmstadt, Germany). Tetramethylsilane (TMS) and pyridine-d_5 (99.5% D) for NMR analysis were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2.2 Extraction and separation of fractions

Raw *P. notoginseng* powder (~600 g) was steamed for 9 h using the method described in Section 3.1.2.3. Steamed *P. notoginseng* powder (230 g) was extracted with methanol (2 L) by ultrasonication for 2 h and filtered under vacuum. This step was repeated for a total of five times. The combined filtrates were evaporated to dryness *in vacuo*, giving a crude methanolic extract (34 g, 15%). The crude residue was dissolved in 140 ml water, washed with 200 ml n-hexane (three times), and extracted with 300 ml water-saturated n-butanol (five times), using liquid-liquid extraction methods. The water, hexane and butanol extracts were evaporated to dryness *in vacuo*. A small portion of each extract was reconstituted with methanol and analysed by HPLC using the same conditions as described in Section 3.1.2.5.

The butanol extract was subjected to multiple normal-phase open-column chromatography (silica gel 60, 63-200 μm particle sizes, 500 length x 30 mm diameter). 4 g of the concentrated butanol extract (dissolved in initial composition of mobile phase) was applied to the column and gradient elution using different compositions of dichloromethane and methanol (9:1, 7:1, 5:1, 7:3, 1:1, 0:1) was employed. The collected fractions were analysed by TLC using dichloromethane and methanol (9:1) as the mobile phase. The spots on the TLC plates were detected by spraying with 40% v/v sulphuric acid in ethanol and heating the plate at 100°C for 10 min. Similar fractions were combined, evaporated, reconstituted and analysed by HPLC using the same HPLC conditions as described in Section 3.1.2.5, except for the shorter gradient: 0-10 min, 20-45% acetonitrile (B); 10-25 min, 45-75% B; 25-28 min, 75-100% B; 28-35 min, 100% B. From the HPLC chromatograms, further combination of similar fractions containing the peaks of interest was carried out. Three main fractions, each containing a pair of the peaks of interest as the major

components, were concentrated to a small volume and subjected to further purification, as described below (Section 4.2.3). The whole column chromatography process was repeatedly performed to obtain sufficient quantities for subsequent studies.

4.2.3 Purification and isolation of pure components

Semi-preparative HPLC methods, using an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) equipped with quaternary gradient pump, autosampler and DAD, were carried out to purify and isolate the pure components. Separation was achieved using semi-preparative reversed-phase column (Agilent Zorbax SB-C18, 250 x 9.4 mm I.D., 5 μm particle size) and isocratic elution using water and acetonitrile. The column temperature was kept constant at 35°C. The UV detection wavelengths were set at 203, 230, 254, 280 nm and DAD was scanned from 190 to 400 nm. Three different isocratic elution profiles (A, B, C) were developed and used, that is, (A) 33% v/v acetonitrile, flow rate was 2.5 ml/min, run time was 25 min, each injection volume was 100 μl; (B) 43% v/v acetonitrile, flow rate was 2.8 ml/min, run time was 25 min, each injection volume was 75 μl; (C) 51% v/v acetonitrile, flow rate was 3 ml/min, run time was 32 min, each injection volume was 150 μl. The fractions were collected by monitoring retention times and peak areas. The tailing portions of each peak were not collected to prevent contamination with close neighbouring peaks. The HPLC runs were repeated until the total volume of the fractions were injected. The collected pure fractions were combined and evaporated to dryness under vacuum.

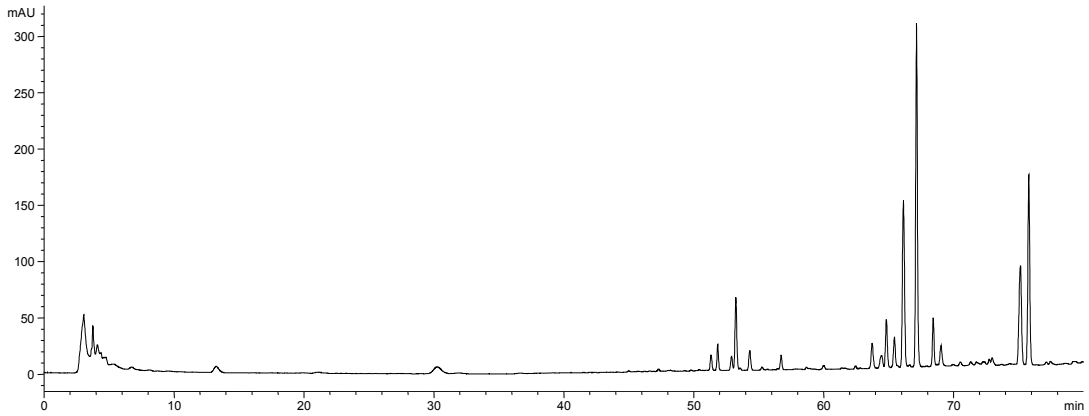
4.2.4 Identification

Melting points were determined with a melting point apparatus (220-240V, Gallenkamp, Loughborough, UK). Mass spectra were obtained using a Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA). The positive mode ESI parameters were set as: sheath gas flow rate 80 units, auxilliary gas flow rate 20 units, spray voltage 4.5kV, capillary temperature 230°C, capillary voltage 10 V, tube lens offset 0 V. Samples dissolved in methanol (~100 μg/ml) were injected directly (5 μl) into the mass spectrometer with 50% v/v ammonium acetate (10 mM) and 50% v/v acetonitrile. The MS data was acquired by Finnigan Xcalibur software. 10-15 mg of the samples was dissolved in pyridine-d_5 and their proton and carbon-13 NMR spectra were acquired at 300MHz on a Bruker AVANCE 300 spectrometer. The chemical shifts (δ) were expressed in parts per million (ppm) relative to TMS as the internal standard.

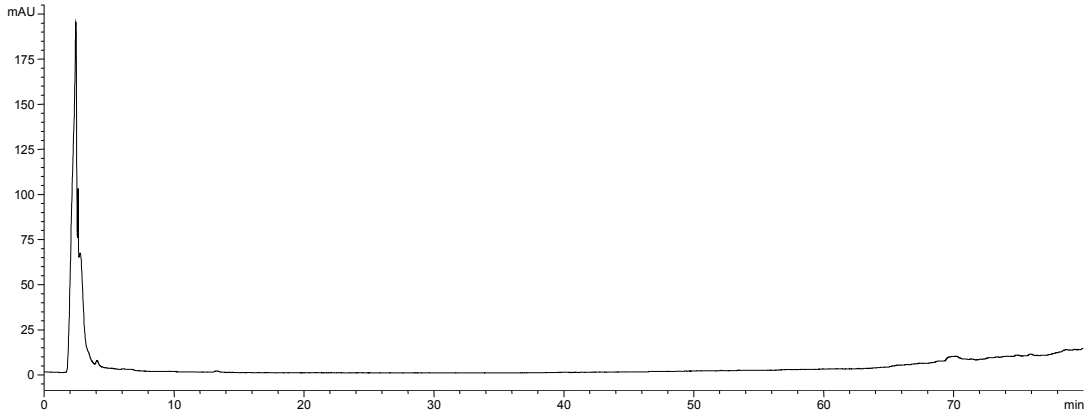
4.3 Results and Discussion

The crude extract was obtained by ultrasonic extraction using methanol and the yield was approximately 15% w/w of the quantity of powdered root used. Initial separation of the crude extract into water, butanol and hexane extracts gave dried yields of 3.7%, 12.3% and 0.3% w/w of the quantity of powdered root (or 24.5%, 82.6% and 2.4% w/w of the dried crude extract) respectively. These corresponded to the polar, moderately polar and non-polar fractions respectively, as seen from the chromatograms of the components present in each of the extracts (Figure 4.1). The hexane extract may consist of non-polar compounds such as lipids, fatty acids, and polyacetylenes. After extraction with n-butanol, the remaining water extract may contain highly polar substances such as polysaccharides, and amino acids that were

(A)



(B)



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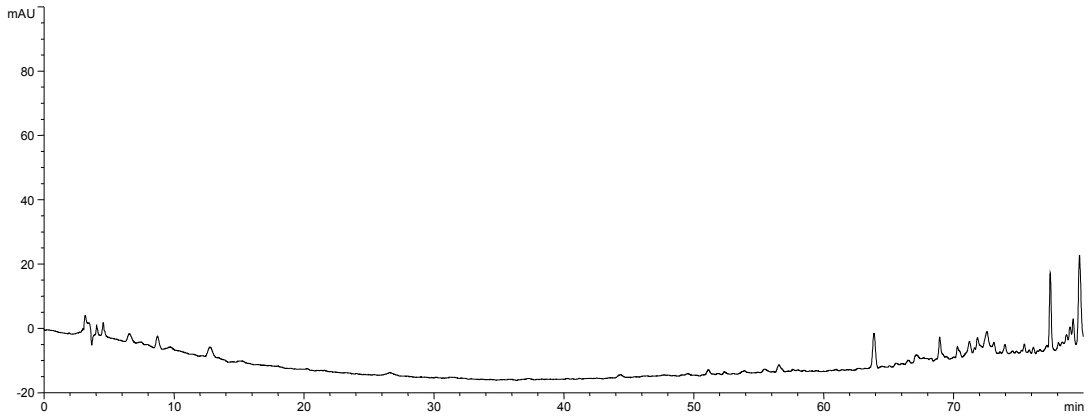
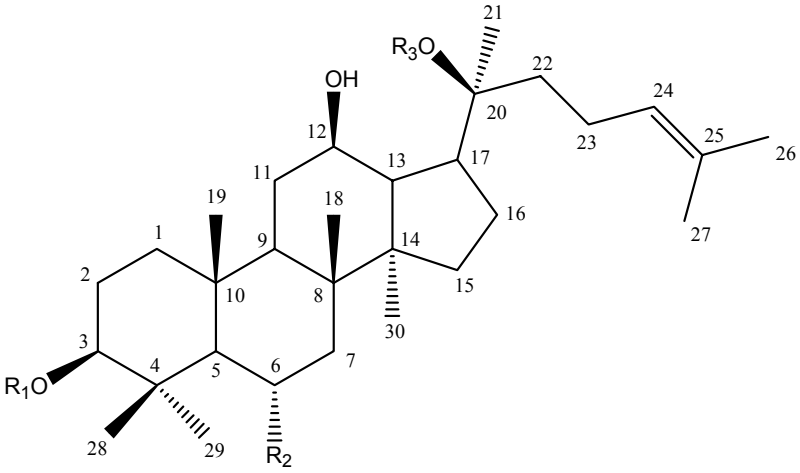
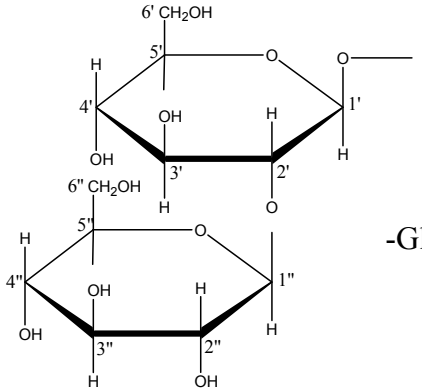


Figure 4.1. HPLC chromatograms of (A) butanol, (B) water, and (C) hexane extracts of steamed *P. notoginsen*g (9 h).

not retained by the reversed-phase column. The HPLC profile of the butanol extract was rather similar to the crude extract except for the large peaks eluting at the solvent front. Therefore, saponins, including the six saponins analysed in Chapter 3, were extracted and found in the butanolic extract. The peaks of interest, which served as differentiating marker compounds to identify the samples, were those present in the steamed form but not detected in the raw form. As these peaks were predominantly in the butanolic extract, therefore subsequent separation and isolation were focused on this particular fraction.

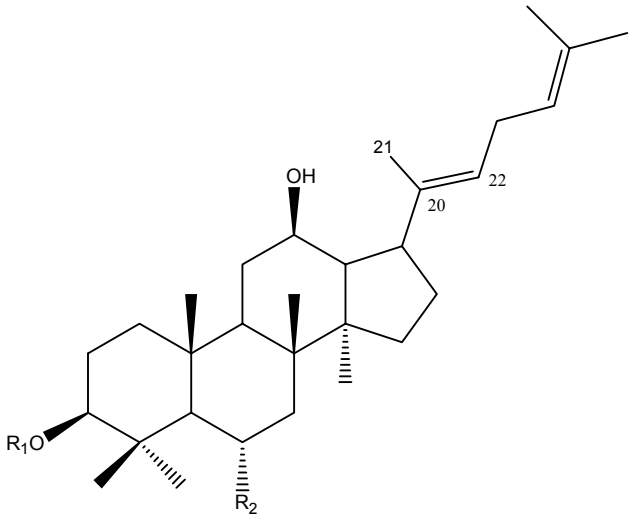
Column chromatography using silica gel was used to fractionate the butanol extract into different fractions, each containing several components. Repeated chromatography was needed to isolate six pure components (U to Z) from complex herbal extracts. Three impure fractions (fraction with U, V: 4.2% w/w of the butanol extract used, fraction with W, X: 3.6% w/w, and fraction with Y, Z: 4.6% w/w), each containing a pair of compounds together with many other minor components, were of interest because they were the differentiating marker compounds for steamed *P. notoginseng*. As the polarities of the pairs of compounds were similar, they eluted together and low resolution normal-phased chromatography was found unsuitable to separate them. Each of the three pairs can only be separated by subsequent semi-preparative HPLC methods, which were optimised to separate the compounds sufficiently to obtain pure compounds. Therefore, the six pure compounds (U to Z) were finally isolated from the butanol extract. Their identities were determined using MS and NMR and the structures are shown in Figure 4.2.



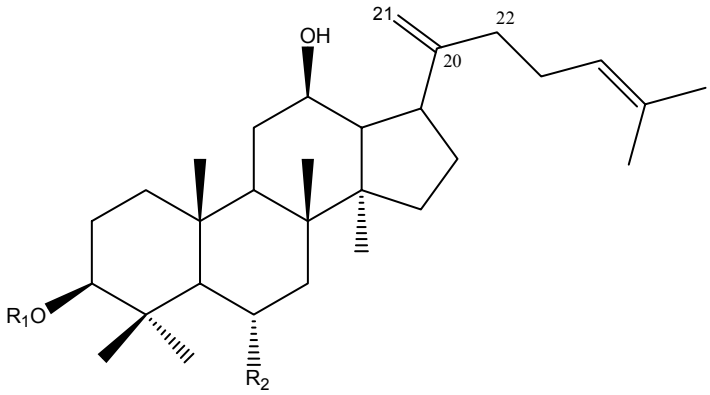


-Glc^{2}-^{1}Glc

Saponins	R_1	R_2	R_3
Protopanaxatriol			
R1	-H	-O-Glc^{2}-^{1}Xyl	-Glc
Rg1	-H	-O-Glc	-Glc
Re	-H	-O-Glc^{2}-^{1}Rha	-Glc
Rg2	**-H**	**-O-Glc^{2}-^{1}Rha**	**-H**
20S-Rh1	**-H**	**-O-Glc**	**-H**
20R-Rh1	**-H**	**-O-Glc**	**-H**
Protopanaxadiol			
Rb1	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Glc
Rc	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Ara(f)
Rd	-Glc^{2}-^{1}Glc	-H	-Glc
20S-Rg3	**-Glc^{2}-^{1}Glc**	**-H**	**-H**
20R-Rg3	**-Glc^{2}-^{1}Glc**	**-H**	**-H**
Rh2	**-Glc**	**-H**	**-H**



Saponins	R_1	R_2
Rg5	**-Glc^{2}-^{1}Glc**	**-H**
Rh4	**-H**	**-O-Glc**



Saponins	R_1	R_2
Rk1	**-Glc^{2}-^{1}Glc**	**-H**
Rk3	**-H**	**-O-Glc**

Figure 4.2. Chemical structures of some saponins present in raw and steamed *P. notoginseng*. Those in bold are characteristic for the steamed samples. Abbreviations: Glc, glucose; Ara(f), arabinose in furanose form; Ara(p), arabinose in pyranose form; Rha, rhamnose; Xyl, xylose.

Compound U: It was obtained as a white amorphous powder with a melting point of 246-248°C (literature: 245-246.5°C) [Ahmad and Basha, 2000]. The yield was 1.6% w/w of butanol extract and purity was >95% as determined by HPLC. UV spectrum was a typical saponin spectrum. Positive ionisation mode ESI-MS revealed a $[M+H]^+$ at *m/z* 639 and $[M+Na]^+$ at *m/z* 661, thereby suggesting a compound with a molecular mass of 638. Product ions at *m/z* 405, 423, and 441 also suggested a protopanaxatriol type of ginsenoside [Miao *et al*., 2002; Chan *et al*., 2000; Wang *et al*., 1999]. Furthermore, a loss of one and two water molecules from the molecular $[M+H]^+$ ion gave rise to product ions at *m/z* 621 and 603 respectively [Wang *et al*., 1999]. The ^{13}C-NMR data were acquired and tabulated in Table 4.1. From comparison with the chemical shifts in the literature for protopanaxatriol, protopanaxadiol and various structurally similar ginsenosides, the signals were assigned. One anomeric carbon signal at 106.01 ppm and signals between 60-80 ppm suggested that the compound has one sugar moiety. Two olefinic carbon signals at 130.81 and 126.31 ppm suggested one double bond, typical of a protopanaxatriol which has a double bond at C-24(25). Its ^{13}C-NMR spectra was found to be similar to ginsenoside Rg1, except that the latter has signals corresponding to two sugar moieties and the signals around C-17, 20, 21 differed [Teng *et al*., 2002], suggesting that the sugar moiety at C-20 may be removed in compound U. Furthermore, the mass difference between compound U and ginsenoside Rg1 was 162, which corresponded to a loss of a glucose unit. The NMR data correlated well with published data [Teng *et al*., 2002], and together with all the above data which supported its identity, compound U was determined to be 3β,6α,12β,20S-tetrahydroxydammar-24-ene-6-O-β-D-glycopyranoside or also named as 20S-ginsenoside Rh1.

Table 4.1. ^{13}C-NMR chemical shifts (δ, ppm) (300MHz, in pyridine-d_5) of compounds U, V, W, X, Y and Z, corresponding to 20S-ginsenoside Rh1, 20R-ginsenoside Rh1, ginsenosides Rk3, Rh4, Rk1 and Rg5 respectively

	Chemical shifts (ppm)					
Carbon positions	20S-Rh1 U	20R-Rh1 V	Rk3 W	Rh4 X	Rk1 Y	Rg5 Z
Aglycone						
1	39.40	39.40	39.53	39.50	39.28	39.31
2	27.88	27.90	27.95	27.92	26.72	28.13
3	78.61	78.61	78.59	78.59	89.09	88.98
4	40.36	40.38	40.41	40.39	39.73	40.28
5	61.43	61.44	61.48	61.44	56.41	56.44
6	80.08	80.10	80.09	80.08	18.44	18.47
7	45.22	45.19	45.35	45.33	35.34	35.37
8	41.09	41.13	41.29	41.35	40.19	39.74
9	50.19	50.21	50.67	50.69	48.23	50.80
10	39.66	39.68	39.74	39.75	37.01	37.06
11	32.04	32.17	32.77	32.30	32.64	32.23
12	71.05	70.95	72.46	72.59	72.48	72.63
13	48.22	48.88	52.10	50.60	52.42	50.46
14	51.65	51.73	51.17	50.86	51.24	51.06
15	31.23	31.34	32.53	32.56	32.64	32.67
16	26.82	26.62	30.74	28.84	30.82	26.78
17	54.73	50.57	48.30	50.42	50.86	50.93
18	17.38	17.41	17.37	17.39	16.46	16.46
19	17.70	17.72	17.77	17.74	15.79	16.62
20	73.04	73.09	155.45	140.11	155.50	140.20
21	27.00	22.74	108.14	13.14	108.15	13.19
22	35.83	43.19	33.73	123.56	33.86	123.22
23	23.00	22.59	27.05	27.46	27.07	27.48
24	126.31	126.03	125.38	123.88	125.36	123.55
25	130.81	130.82	131.22	131.26	131.26	131.29
26	25.83	25.84	25.78	25.70	25.78	25.70

27	17.64	17.72	17.37	17.74	17.77	17.73
28	31.72	31.73	31.73	31.73	28.09	28.87
29	16.39	16.40	16.38	16.38	16.59	15.85
30	16.78	17.08	16.76	16.81	16.99	17.05
Sugar moieties						
6-Glc						
1'	106.01	106.00	106.03	106.02		
2'	75.47	75.49	75.48	75.47		
3'	79.62	79.65	79.68	79.66		
4'	71.84	71.85	71.84	71.83		
5'	78.13	78.17	78.17	78.16		
6'	63.05	63.08	63.09	63.08		
3-Glc						
1'					105.10	105.13
2'					83.04	83.42
3'					78.12	78.27
4'					71.58	71.66
5'					77.98	78.00
6'					62.75	62.77
1"					105.79	106.02
2"					77.00	77.12
3"					78.34	78.38
4"					71.70	71.72
5"					77.98	78.12
6"					62.75	62.88

Compound V: It was obtained as a white amorphous powder with a melting point of 155-158°C (literature: 154-158°C). The yield was 0.5% w/w of butanol extract and purity was >95% as determined by HPLC. The positive ionisation mode ESI mass spectrum was similar to that of compound U, with $[M+H]^+$ at *m/z* 639 and $[M+Na]^+$ at *m/z* 661, thereby suggesting that the two compounds, U and V, were isomers with a similar molecular mass of 638. The NMR signals of compounds U and V were almost identical, except for the slight differences in carbon signals at three carbon positions. As seen from Table 4.1, the differences in NMR chemical shifts between compounds U and V were mainly at C-17 (+4.16 ppm), C-21 (+4.26 ppm) and C-22 (-7.36 ppm). They were likely to be structurally similar. From comparison with all published literature and the above deductions, the results obtained in this study was found to correlate well with previously reported data [Teng *et al*., 2002] of 3β,6α,12β,20R-tetrahydroxydammar-24-ene-6-O-β-D-glycopyranoside or also known as 20R-ginsenoside Rh1. Therefore, compounds U and V are epimers of each other and the change in configuration at C-20 has mainly affected the chemical shifts of the neighbouring carbons (C-17, C-21, C-22). Similar changes at these positions were observed for another pair of 20S and 20R-ginsenoside Rg3 [Teng *et al*., 2000].

Compound W: It was obtained as a white amorphous powder with a melting point of 146-148°C (literature: 145-147°C). The yield was 1.0% w/w of butanol extract and purity was >96% as determined by HPLC. Positive ionisation mode ESI-MS revealed a $[M+H]^+$ at *m/z* 621 and $[M+NH_4]^+$ at *m/z* 638, thereby suggesting a compound with a molecular mass of 620. From the ^{13}C-NMR data (Table 4.1), one anomeric carbon signal at 106.03 ppm and signals between 60-80 ppm suggested that the compound has one sugar moiety. However, there were four olefinic carbon signals at 155.45, 131.22, 125.38, 108.14 ppm which suggested two double bonds. The

signals were quite similar to that of ginsenoside Rh3 (molecular weight = 604), except for one additional hydroxyl group (16 mass units), and it is likely to be protopanaxatriol type ginsenoside. The ^{1}H and ^{13}C-NMR data correlated well with a literature report (Park *et al*., 2002), and compound W was determined to be 3β,6α,12β-trihydroxydammar-20(21),24-diene-6-O-β-D-glycopyranoside or otherwise named as ginsenoside Rk3.

Compound X: It was obtained as a white amorphous powder with a melting point of 156-158°C (literature: 155-157°C). The yield was 1.0% w/w of butanol extract and purity was >96% as determined by HPLC. The mass spectrum showed a $[M+H]^+$ at *m/z* 621 and $[M+NH_4]^+$ at *m/z* 638, thereby suggesting a compound with a molecular mass of 620 and may be an isomer of compound W. The ^{13}C-NMR data signals (Table 4.1) were similar to that of compound W, except for some of the olefinic carbon signals, suggesting that the double bonds were at different positions. Furthermore, these olefinic signals were similar to that of ginsenoside Rh3, suggesting similar double bond positions. The ^{1}H and ^{13}C-NMR data correlated well with the data in a previous report [Park *et al*., 2002b], and together with the above supporting data, compound X was therefore identified to be 3β,6α,12β-trihydroxydammar-20(22),24-diene-6-O-β-D-glycopyranoside or also known as ginsenoside Rh4.

Compound Y: It was obtained as a white amorphous powder with a melting point of 177-180°C (literature 178-181°C). The yield was 0.4% w/w of butanol extract and purity was >95% as determined by HPLC. The molecular mass was determined to be 766 from the positive mode ESI-MS which showed a $[M+H]^+$ ion at *m/z* 767, $[M+NH_4]^+$ at *m/z* 784 and $[M+Na]^+$ at *m/z* 789. From the ^{13}C-NMR data (Table 4.1), two anomeric carbon signals at 105.10 and 105.79 ppm and at least ten

signals between 60-90 ppm suggested that the compound has two sugar moieties. Four olefinic carbon signals at 155.50, 131.26, 125.36, 108.15 ppm also suggested the presence of two double bonds. The molecular mass was 18 units less than ginsenoside Rg3 (molecular mass = 784) and was likely to be its dehydrated compound. The NMR signals were rather similar to ginsenoside Rg3 except for the number of olefinic carbon signals, suggesting that it has one more double bond. The methyl carbon signal from C-21 was not observed in compound Y, but a methylene carbon signal at 108.15 ppm was detected instead. The ^{1}H and ^{13}C-NMR data correlated well with previous report [Park *et al*., 2002], and compound Y was deduced to be 3β,12β-dihydroxydammar-20(21),24-diene-3-O-β-D-glycopyranosyl (1→2)-β-D-glucopyranoside or also named as ginsenoside Rk1.

Compound Z: It was obtained as a white amorphous powder with a melting point of 188-191°C (literature: 188-192°C). The yield was 0.5% w/w of butanol extract and purity was >97% as determined by HPLC. It is an isomer of compound Y with a similar molecular mass of 766 due to a $[M+H]^+$ at *m/z* 767, $[M+NH_4]^+$ at *m/z* 784 and $[M+Na]^+$ at *m/z* 789. The ^{13}C-NMR data signals (Table 4.1) were similar to that of compound Y, except for some of the olefinic carbon signals, suggesting that the double bonds were at different positions. These olefinic signals were the same as that of ginsenoside Rh3, implying that the double bond positions were at similar positions. However, compound Z has another glucose moiety compared to ginsenoside Rh3. The ^{1}H and ^{13}C-NMR data correlated well with previous report [Kim *et al*., 1996] and together with the above supporting data, compound Z was found to be 3β,12β-dihydroxydammar-20(22),24-diene-3-O-β-D-glycopyranosyl (1→2)-β-D-glucopyranoside or also known as ginsenoside Rg5.

Besides the above six compounds, peaks S and T in the HPLC chromatograms (Figure 3.6C in Chapter 3) were also increased in *P. notoginseng* samples that were steamed for 9 h. They were identified as 20S-ginsenoside Rg3 (S) and 20R-ginsenoside Rg3 (T) respectively (Figure 4.3). Their identities were confirmed by comparing their retention times, UV spectra, MS spectra with those of the standards, as well as spiking of the standards into the steamed extracts. 20S and 20R-ginsenosides Rg3 have similar mass spectra. Similar to 20S- and 20R-ginsenoside Rh1, it can be seen that 20S-ginsenoside Rg3 was eluted earlier and it was present in higher quantities than its corresponding 20R-epimer. Ginsenosides Rk3 and Rk1 (Δ20(21)) were also eluted earlier than its Δ20(22) isomers (ginsenosides Rh4 and Rg5). Moreover, 20RS-ginsenosides Rg2 and 20S-ginsenoside Rh2 were detected in low quantities in steamed *P. notoginseng* crude extracts. These components were also detected in red *P. ginseng* [Li, 1986; Kim *et al*., 2000; Kitagawa *et al*, 1987].

All of these compounds (S to Z) were not detected in raw samples, so the steaming process has caused chemical degradation and conversion of some saponins to new components. From the structures, it can be seen that 20S- and 20R-ginsenoside Rg3 are most likely produced by the loss of glycosyl moiety at C-20 position of protopanaxadiol type saponins (e.g. ginsenosides Rb1, Rc, Rd). 20RS-ginsenoside Rg2 may arise from protopanaxatriol type saponins (e.g. ginsenoside Re) in the same manner. Upon steaming, isomerisation of the hydroxyl configuration at C-20 gives rise to the S and R isomers. Similarly, it is proposed that 20S- and 20R-ginsenosides Rh1 may arise from protopanaxatriol type saponins (e.g. ginsenoside Rg1) by the loss of glycosyl moiety at C-20. Alternatively, they may also be formed by the loss of C-20 glucose and one rhamnose sugar moiety at C-3 of ginsenoside Re. Ginsenosides Rg5 and Rk1 are postulated to be produced by further dehydration at C-20(22) and C-

20(21) of ginsenoside Rg3 respectively. Similar dehydration of ginsenoside Rh1 at C-20 can also give rise to ginsenosides Rh4 and Rk3. Therefore, these explain the changes in components and the reasons for the decrease in concentrations (as shown in Chapter 3) of some of the saponins upon steam processing.

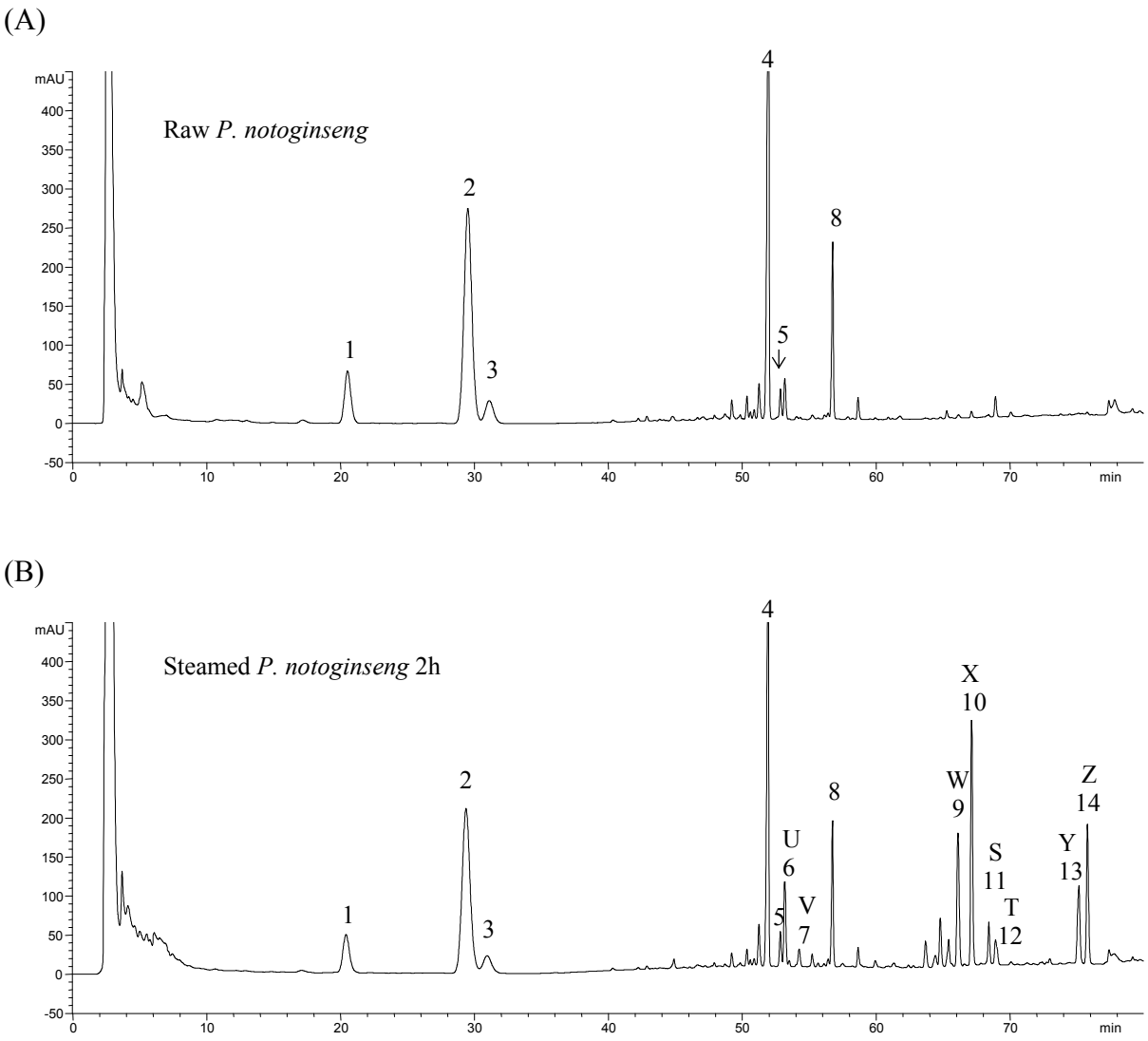


Figure 4.3. HPLC chromatograms of (A) raw and (B) steamed *P. notoginseng* (2 h). (1)R1, (2)Rg1, (3)Re, (4)Rb1, (5)Rc, (6)20S-Rh1, (7)20R-Rh1, (8)Rd, (9)Rk3, (10)Rh4, (11)20S-Rg3, (12)20R-Rg3, (13)Rk1, (14)Rg5. Peaks 6, 7, 9, 10, 11, 12, 13 and 14 corresponded to peaks U, V, W, X, S, T, Y and Z in Figure 3.6C respectively.

4.4 Conclusion

Eight major potential markers (20S-ginsenoside Rh1, 20R-ginsenoside Rh1, 20S-ginsenoside Rg3, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5) that play key roles in differentiating the chromatograms of raw and steamed *P. notoginseng* were successfully identified in this study. This is the first report of the detection and isolation of ginsenosides Rk1 and Rk3 from *P. notoginseng* roots, as well as the first separation of these fourteen main saponins in a single HPLC chromatogram of steamed *P. notoginseng*. These biomarkers are potentially useful for the quality control of raw and steamed *P. notoginseng* roots and their products.

CHAPTER 5

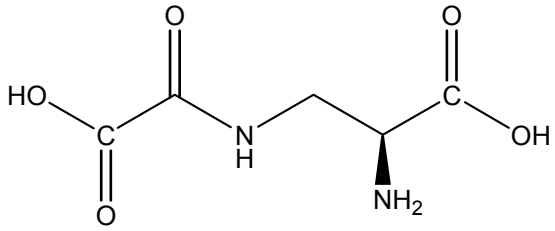
LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC ANALYSIS OF DENCICHINE IN *PANAX NOTOGINSENG* AND RELATED SPECIES

5.1 Introduction

Dietary non-protein amino acids have been implicated as potential factors interfering with fundamental biochemical processes and causing clinical disorders [Rubenstein, 2000]. The naturally occurring β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Figure 5.1A), a neuro-excitatory non-protein amino acid, was first isolated from the seeds of *Lathyrus sativu*s (grass pea seeds) [Rao *et al*., 1964]. It is also known as dencichine. It was identified as the main cause of a primary upper motor neuron degenerative disease known as neurolathyrism, a condition with symptoms such as hyper-irritability, severe weakness and irreversible paralysis of the leg muscles, and convulsions. This disease is prevalent in endemic form, often occurring after drought-induced famines in the Indian subcontinent or in Ethiopia, when grass pea seed becomes the exclusive or main dietary staple for several months. Besides being present in the seeds of 21 *Lathyrus* species, β-ODAP was found in other genera of leguminous plants including *Acacia* and *Crotalaria* species [Quereshi *et al*., 1977].

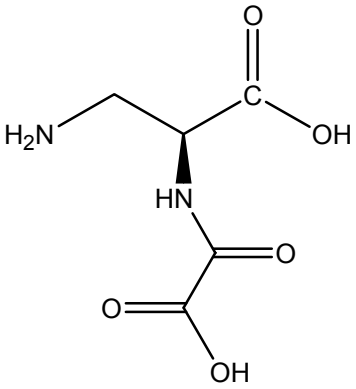
The mechanisms whereby β-ODAP causes neurotoxicity have been researched by some investigators. It showed strong agonistic activity on recombinant AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and has facilitating and/or prolonging effects on glutamatergic transmission by inhibition of glutamate

(A)



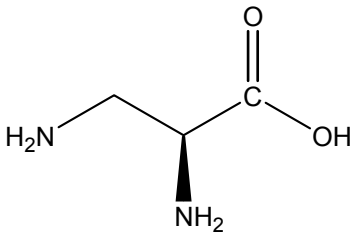
Mol. Wt: 176

(B)



Mol. Wt: 176

(C)



Mol. Wt: 104

Figure 5.1. Chemical structures of (A) dencichine, i.e., β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP), (B) α-*N*-oxalyl-L-α,β-diaminopropionic acid (α-ODAP), and (C) α,β-diaminopropionic acid (DAP).

transport [Kuo *et al*., 2003]. Mitochondrial dysfunction mediated by β-ODAP was also suggested as an underlying cause of neurolathyrism [Ravindranath, 2002].

Interestingly, dencichine is also found to be present in *Panax* species, the only non-leguminous plants in which β-ODAP is known to be present [Kuo *et al*., 2003]. Furthermore, it is a bioactive therapeutic amino acid component in *P. notoginseng*. Studies have reported dencichine as the component responsible for the herb's main haemostatic and platelet increasing properties *in vivo* [Zhao and Wang, 1986]. The exact mechanisms whereby dencichine causes haemostatic effects are still not known. The haemostatic effect was present at a low dose of dencichine [Zhao and Wang, 1986], while neurotoxicity occurred at higher doses. Medicinal herbs or tonics, when used appropriately, are usually not consumed in quantities as high as staple food like the *Lathyrus* species. Therefore the possibility of developing neurolathyrism from over-consumption of such herbs is very much lower. The amount of dencichine in raw *P. notoginseng* roots [Zhang *et al*., 1990; Dong *et al*., 2003; Long *et al*., 1996], *P. ginseng* [Kuo *et al*., 2003; Long *et al*., 1996; Zheng and Li, 1990] and *P. quinquefolium* [Zheng and Li, 1990] have been reported. However, to our knowledge, the concentration of dencichine present in steamed *P. notoginseng* has not been previously reported prior to this work. Since the raw root but not the steamed root is used to promote haemostatic effects, it is postulated that the steamed root may have a lower concentration of dencichine. However, to test this postulation, an accurate, sensitive and specific analytical method must be developed.

Due to the concerns regarding neurotoxicity, previous methods developed for the analysis of β-ODAP have focused mainly on the *Lathyrus* species. In the earlier studies the most common approach was a colorimetric method which utilised the reaction of o-phthalaldehyde (OPT) with α,β-diaminopropionic acid, a hydrolysis

product of β-ODAP [Rao, 1978; Briggs et al., 1983]. Dencichine in raw *P. notoginseng* root was also determined by HPTLC with fluorometric detection [Zhang *et al*., 1990]. As dencichine has weak UV absorption and high polarity, various derivatisation methods have been adopted for reversed-phase HPLC analysis. Pre-column derivatisation methods involved phenyl isothiocyanate [Kuo *et al*., 2003; Khan *et al*., 1994; Khan *et al*., 1993], 9-fluorenyl methylchloroformate [Geda *et al*., 1993], 1-fluoro-2,4-dinitrobenzene [Wang *et al*., 2000b] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [Chen *et al*., 2000]. A GC/MS method [Pan *et al*., 1997] using N-ethoxycarbonyl ethyl ester derivatives was also reported. However, all the above methods required derivatisation steps and were laborious, inconvenient and time-consuming. Other potential problems include poor derivative stability, side reactions, reagent interferences and reagent toxicities. An alternative and relatively simple but less sensitive capillary electrophoresis method [Arentoft and Greirson, 1995; Zhao *et al*., 1999a] without derivatisation was subsequently developed. Alternative detection methods such as electrochemical and direct low-wavelength UV detection [Dong *et al*., 2003] have also been proposed. Novel LC-Biosensor systems [Moges and Johansson, 1994; Belay *et al*., 1997a; Yigzaw *et al*., 2001] allowed selective analysis of β-ODAP, but they involved complicated procedures such as the preparation of bienzyme-modified electrode.

LC/MS is an important analytical method due to its high speed, specificity, selectivity and sensitivity. It provides additional structural information and is especially useful for complex samples where chromatographic resolution is limited. LC/MS analyses of small protein amino acid molecules without derivatisation [Petritis *et al*., 2000; Qu *et al*., 2002; Nagy *et al*., 2003; Desai and Armstrong, 2004] were advantageous, but difficulties existed in both the chromatographic retention and

detection of these polar hydrophilic compounds that lack chromophoric groups. Most of the current HPLC methods for analysis of protein amino acids utilised volatile ion-pairing reagents [Petritis *et al*., 2000; Qu *et al*., 2002] to retain the underivatised amino acids on the reversed-phase column.

Due to the high polarity and low molecular weight of dencichine, it is a challenge to analyse it using HPLC or LC/MS. To date, to our knowledge, no LC/MS method has been reported for the analysis of dencichine prior to this current work [Koh *et al*., 2005]. Therefore, the present chapter aims to develop a LC/MS/MS method for the quantification of underivatised dencichine in complex plant matrices of *Panax* species. The developed method will be used to determine if dencichine is present at different levels in raw and steamed *P. notoginseng* samples. The dencichine concentrations of these samples will also be compared with those present in two other *Panax* species, i.e., *P. ginseng* and *P. quinquefolium*.

5.2 Experimental

5.2.1 Materials

Dencichine standard was obtained from Lathyrus Technologies (Hyderabad, India). Diaminopropionic acid (99% purity) was obtained from Bachem AG (Bubendorf, Switzerland). The water was deionised and purified by Milli-Q Gradient A-10 water purification system (Millipore, Molsheim, France). HPLC-grade acetonitrile and methanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). Analytical-grade ammonium formate (97% purity), HPLC-grade ammonium acetate (≥ 99% purity) and mass spectrometry-grade formic acid (98%) reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The raw *P. notoginseng* roots (Samples 1-7) and the reference raw *P. notoginseng* roots (Sample 8) were obtained

from different sources, as mentioned in Section 3.1.2.1. Eleven pairs of raw and steamed *P. notoginseng* CPMs were shown in Table 3.1. Samples of *P. ginseng*, *P. quinquefolium* and Korean red ginseng were also used (Section 3.1.2.1).

5.2.2 Sample and standard preparation

The roots were ground into powder using a laboratory blender. A total of 100 mg of each powdered sample were accurately weighed and extracted with 1 ml of water by ultrasonication for 2 h. The extracts were centrifuged at 20,000 *g* for 20 min. The supernatants were then diluted with water/ acetonitrile (40:60) and filtered through a 0.45 μm nylon filter membrane prior to analysis.

Samples of a powdered raw *P. notoginseng* root were steamed at 120°C for 2, 6 and 9 h respectively (Section 3.1.2.3). The steamed powders were then extracted using ultrasonication as described above. A 1 mg/ml dencichine standard stock solution in water was prepared. For the calibration curves, the stock solution was diluted with water/ acetonitrile (40:60) to give five different concentrations ranging from 2 to100 ng/μl.

5.2.3 HILIC/ESI-MS/MS

A Micromass Quattro micro API triple-quadrupole mass spectrometer, equipped with a Z-spray electrospray source, was interfaced with a Waters Alliance 2695 separation module (Milford, MA, USA). Chromatography was performed on a Waters Atlantis HILIC silica (50 x 2.1 mm I.D., 3 μm) column. The mobile phases used were: (A) 200 mM ammonium formate (pH 3.0) and (B) acetonitrile; isocratic elution using 40% v/v A and 60% v/v B was employed for dencichine. The total run

time was 5 min. Column temperature was kept constant at 30°C. The flow rate was 0.3 ml/min and the injection volume was 2 µl. Due to the relatively low flow rate, the LC effluent entered the mass spectrometer interface directly, without splitting.

The mass spectrometer was operated in positive electrospray ionisation (ESI) mode. The mass scale of the instrument was calibrated using a solution of sodium and caesium iodides. Tuning was performed by direct infusion of a solution of dencichine in 40% v/v mobile phase A plus 60% v/v acetonitrile at a flow rate of 5 µl/min, using the built-in syringe pump. Nitrogen gas was used as the desolvation gas at a flow rate of 500 L/h. The following optimised MS tune parameters were used: ESI capillary, 3.0 kV; cone, 18 V; extractor, 3.0 V; RF lens, 0 V; source temperature, 120°C; desolvation temperature, 300°C; resolution (LM1, HM1, LM2, HM2), 15.0; ion energy 1, 0.2; ion energy 2, 1.0; entrance, -1; exit, 3; multiplier, 650 V. MS/MS experiments were performed using argon as the collision gas. The collision energy was optimised to give the highest product ion intensities; the optimal collision energy was 12 eV. Dencichine was analysed and quantified in the multiple reaction monitoring (MRM) mode using the transition *m/z* 177 → 116. The dwell time was 0.5 s, and the interscan delay was 0.05 s. Data were acquired and processed using MassLynx^{TM} version 4.0 software (Waters, Milford, MA, USA).

5.2.4 Method validation

To assess linearity, the calibration curves were analysed by linear least-squares regression analysis. The LOD and LOQ were obtained by diluting the dencichine standard until the signal-to-noise (S/N) ratio reached 3:1 and 10:1, respectively. The injection/instrument precision (calculated as relative standard

deviation, RSD), for a low concentration of dencichine at the LOQ limit, was determined by six consecutive injections. For intra-day assay precision, five concentrations of dencichine standard were each assayed five times within a day. For inter-day assay precision, triplicate analyses of five concentrations of dencichine were performed on three different days. Recoveries (accuracies) of dencichine from the plant matrix were determined by spiking dencichine at 3 different levels (0.2, 0.15, 0.09 mg/ml) into raw *P. notoginseng* samples before extraction and then experimentally measuring the added amounts. A calibration curve was acquired each day, before the analysis of each set of samples, to ensure accuracy. Blanks (40% v/v water plus 60% v/v acetonitrile) and a quality control standard (5 ng/μl of dencichine) were run intermittently between the samples to verify the instrument response.

5.3 Results and Discussion

5.3.1 Optimisation of MS/MS conditions

Positive mode ESI spectra of dencichine contained the $[M+H]^+$ ion at *m/z* 177. The product ion scans of *m/z* 177 indicated fragmentations dominated by product ions at *m/z* 160, 159, 131, 116, 105, 88, 87, 70, 59 (Figure 5.2). MS/MS is preferable to MS for detection because of the higher sensitivity (S/N ratio) and selectivity, especially when dencichine is present in complex matrices. Selection of a proper transition for MS/MS detection of an analyte is based on two main factors. Firstly, the transition should be specific to the compound, and secondly, the product ion should be sufficiently abundant for sensitive detection. The transition from *m/z* 177 $\rightarrow$ 116 was chosen based on these criteria. Flow injection analysis of dencichine produced stable total ion currents for the chosen MRM transition. Blank injection analysis also excluded the possibility that any background compound would produce a response in

the chosen MRM transition. Therefore, this transition *m/z* 177 → 116 was used for the subsequent analysis of dencichine in the samples.

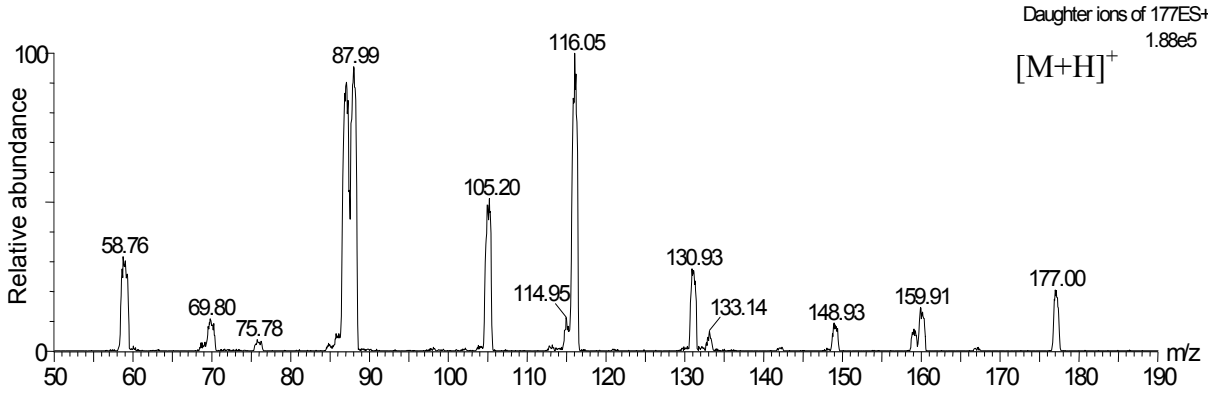


Figure 5.2. MS/MS product ion spectrum of the $[M+H]^+$ ion of dencichine obtained using direct syringe infusion.

A postulated fragmentation pathway of protonated dencichine is as follows. The product ions *m/z* 160 and 159 correspond to losses of NH_3 and of water, respectively. Loss of CO from *m/*z 159 results in the formation of an immonium ion (*m/z* 131). The key product ion at *m/*z 116 probably arises from protonation at the primary amine group followed by loss of NH_3 and then rearrangement of the resulting carbonium ion to lead to loss of CO_2. The ion at *m/z* 105 probably corresponds to protonated α,β-diaminopropionic acid, resulting from initial protonation on the amide nitrogen followed by an H-atom transfer and losses of CO_2 and CO. Further losses of ammonia, water or CO_2 from *m/z* 105 give rise to the product ions at *m/z* 88, 87 and 59, respectively.

The various MS/MS parameters were optimised for sensitivity by experiments using syringe infusion. Both the cone voltage and collision energy have important influences on the product ion intensity (Figure 5.3). By modifying the cone voltage and collision energy, the product intensity fluctuated and achieved optimised level at 18 V and 12 eV, for cone voltage and collision energy, respectively. These optimised parameters were used throughout the experiments.

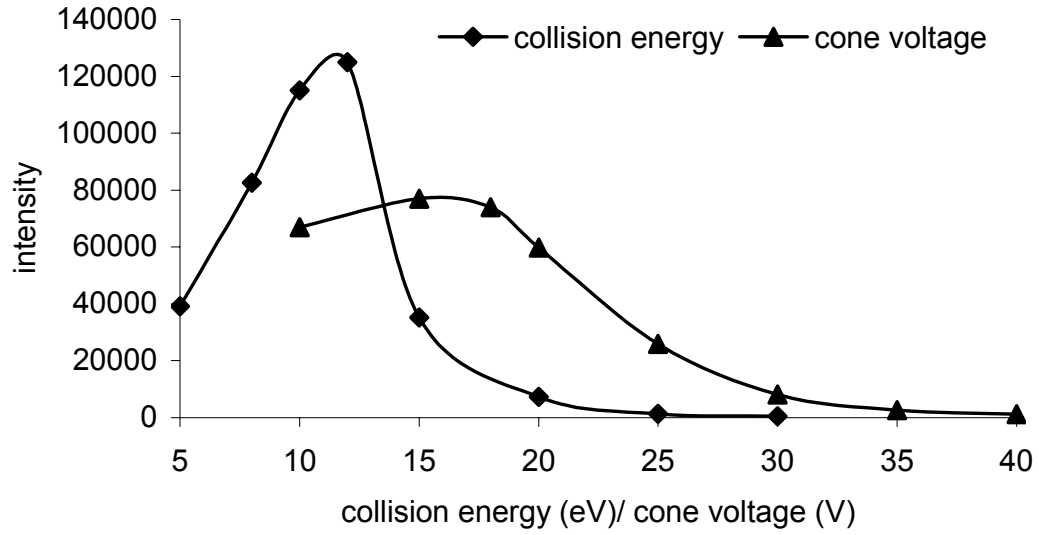


Figure 5.3. Product ion intensity (*m/z* 116) of dencichine versus changes in collision energy and cone voltage.

5.3.2 Optimisation of LC conditions

Several C_{18} reversed-phase columns were tested during method development and results showed that dencichine was not retained by any of the reversed-phase packing. It was eluted in less than 0.45 min in the void volume of these columns, despite a highly aqueous condition of 95-100% v/v water or buffer. Under such highly aqueous conditions the MS sensitivity may be compromised. Besides derivatisation,

other options for retaining polar analytes include the use of ion exchange and ion pairing chromatography. Ion exchange chromatography often involves solvents with high concentrations of non-volatile inorganic salts, which is incompatible with coupling to MS. Ion pairing chromatography was explored in the preliminary study. 0.1% trifluoroacetic acid (TFA), a commonly used volatile mobile phase modifier used for peptides and proteins, was found unable to retain dencichine. Furthermore, a disadvantage of ion pairing agents such as TFA is the possible ion suppression in ESI-MS. Another common alternative to retain polar compounds is the manipulation of pH to make the compounds neutral. Despite the use of various types of buffers with different pH range, dencichine was not retained. This is due to the fact that dencichine can undergo three acid dissociation steps, similar to glutamic acid and aspartic acid. The pKa values 1.95, 2.95 and 9.25, corresponding to the two carboxylic and α-amino functions respectively, are very different. Therefore, in the pH range of most usable buffer systems, the compound will be in the charged zwitterionic form (all three groups ionised).

Hydrophilic Interaction Chromatography (HILIC) was subsequently found to retain dencichine and was selected for the present work on dencichine. HILIC is a type of separation technique which was first described by Alpert in 1990 [Alpert, 1990], consisting of a hydrophilic stationary phase that is eluted with a more hydrophobic mobile phase so that retention times increase with the hydrophilicity of the solutes. It is a variant of normal phase chromatography in which the presence of water in the mobile phase is crucial for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which analytes may selectively partition. Retention involves a mixed mode mechanism involving both partitioning and cation exchange mechanisms. With its high organic content mobile

phases, HILIC also promotes enhanced MS response. Applications of this separation technique to highly polar compounds have been reported previously [Alpert, 1990; Strege, 1998; Schlichtherle-Cerny *et al*., 2003; Tolstikov and Fiehn, 2002].

Further optimisation of several conditions such as injection volume, flow rate, pH, and mobile phase composition, was then performed to reduce the peak width, as the peak was initially broad. Typically, the use of buffers can affect the electrostatic interactions between the solute and stationary phase, and changing the buffer concentration had the most significant effect on the peak shape. It was found that by increasing the buffer concentration (10, 20, 40, 50, 60, 70, 80 mM), the peak was sharpened. 80 mM of ammonium formate on-column gave optimal results, and still permitted sufficient MS sensitivity. Ammonium acetate and ammonium formate buffers were compared at the same concentration; the nature of the buffer anions had minimal effects on the dencichine peak. A change in pH (pH 2.75 to 4.75) did not have much effect on the peak shape, i.e., the method was robust with respect to slight variations in pH. Increasing the acetonitrile composition, however, increased the retention time; with 60% v/v acetonitrile and 40% v/v aqueous buffer, the retention time for dencichine was about 1 min, which was beyond the void volume of the column used to prevent any ion suppression by early eluting polar compounds.

The complex samples were analysed by HILIC/MS/MS in the MRM mode and only one peak was found, showing high selectivity of the method. Furthermore, this rapid elution of 1 min is advantageous for the high-throughput analysis of numerous samples. The LC/MRM chromatograms for dencichine standard and for the extracts of raw and steamed *P. notoginseng* samples are presented in Figures 5.4A to 5.4C.

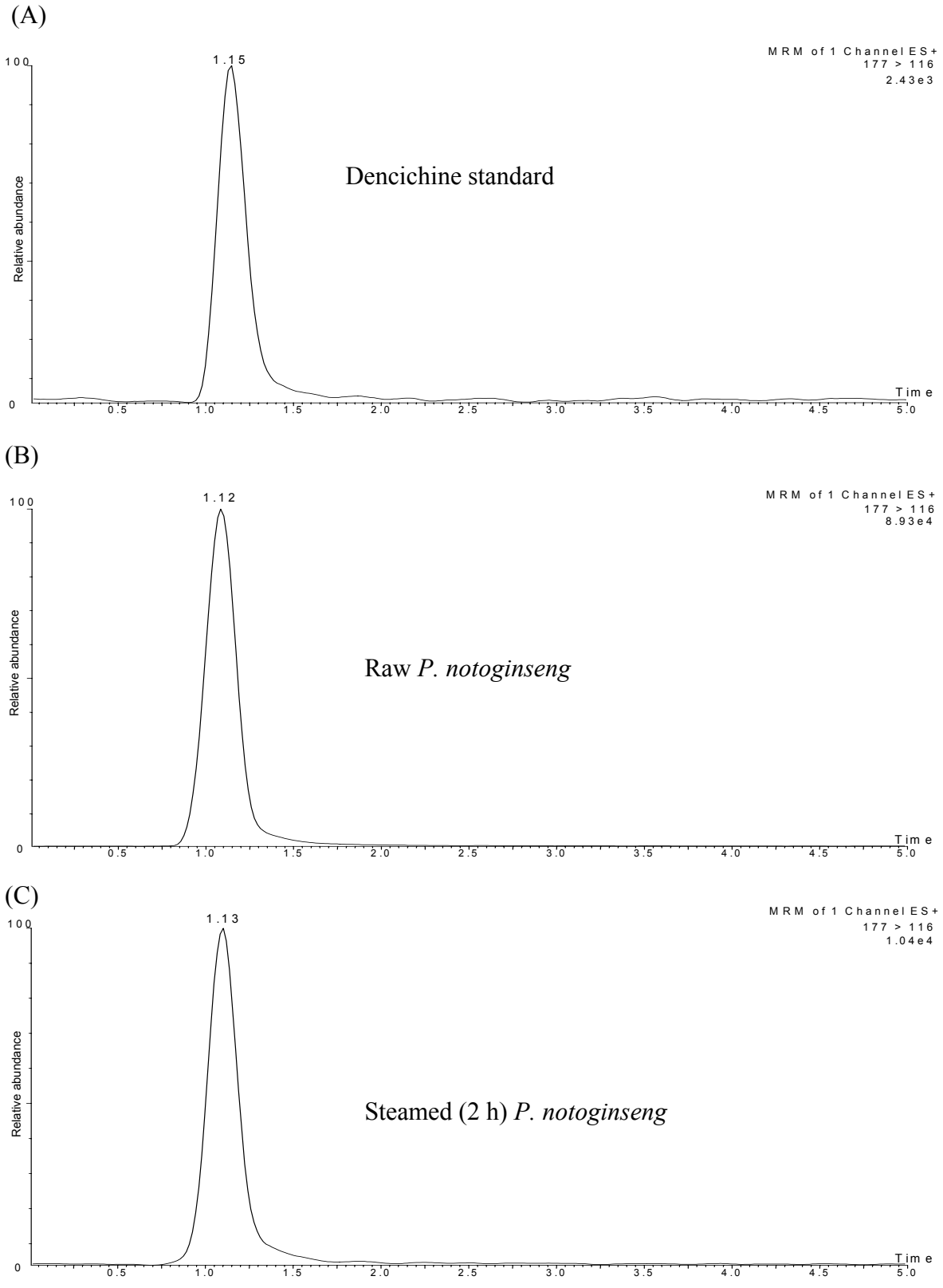


Figure 5.4. LC/MRM chromatograms of (A) 10 ng/μL dencichine standard, (B) extract of raw *P. notoginseng* sample, and (C) extract of steamed (2 h) *P. notoginseng* sample. The MRM transition was *m/z* 177 → 116.

5.3.3 Optimisation of sample preparation

Previous studies have used several different methods and solvents to extract dencichine from *Lathyrus sativus* and *Panax* species. To experimentally determine the optimum solvents for extraction of dencichine from the *P. notoginseng* samples, each of two different raw *P. notoginseng* samples was extracted using 100% methanol, 70% v/v, 50% v/v, and 30% v/v aqueous methanol, and 100% water. Peak areas of dencichine in the samples increased with increasing proportions of water, for example, it increased several folds from 1250 (100% methanol) to 14236 (100% water) for one of the samples. The results showed that 100% water consistently gave higher extraction yield of dencichine than the different mixtures of aqueous methanol. This was within expectations, as dencichine showed much better solubility in water than in methanol. Although the type of solvents used in previous studies varies and includes 30-75% aqueous methanol/ethanol [Rao, 1978; Briggs *et al*., 1983; Wang *et al*., 2000b; Chen *et al*., 2000; Zhao *et al*., 1999a], water [Dong *et al*., 2003; Long *et al*., 1996; Geda *et al*., 1993] and aqueous buffer (pH 7-7.5) [Moges and Johansson, 1994; Belay *et al*., 1997b; Yigzaw *et al*., 2001], but water has been selected based on the results of this study.

Among the wide variety of extraction methods, such as homogenisation, ultrasonication, agitation with a magnetic stirrer, mechanical shaking, rotation in an oven, and overnight maceration, ultrasonication was found to be useful in reducing the time required for extraction and increasing the extraction efficiency. This method was employed in this study. Ultrasonication duration of 1 h and 2 h were also compared in the preliminary study. It was found that 2 h extraction gave a slightly higher amount of dencichine. However, repeating the ultrasonication process three

times and concentrating to the same volume made a negligible difference to the amount of dencichine extracted.

This sample preparation method was simple and rapid compared to methods requiring derivatisation. This is definitely advantageous for preparation of a large number of samples. Further purification of the complex samples is not necessary as MRM is highly selective. Sufficient sensitivity was obtained for the analysis of the samples, despite the complex plant matrices.

5.3.4 Method validation

The LOD (S/N = 3) and LOQ (S/N = 10) for dencichine were found to be 0.3 ppm (or 0.6 ng on column) and 1.5 ppm (or 3 ng on column) respectively. The calibration curves constructed for the assay of dencichine showed good linearity over the concentration range 2 to 100 ng/μl with correlation coefficient $r^2 = 0.9996$ (y = 129.26x – 201.93). The inter-day retention time precision (RSD) was 2.1%. The injection precision (RSD) for a low concentration of dencichine standard (at the LOQ limit, 1.5 ppm) was 6.7% (n = 6). The intra-day and inter-day assay precisions (RSD) of dencichine for the concentration range of 2 to 100 ng/μl were 1.7-4.6% (n = 5) and 3.3-7.9% (n = 3) respectively. The values reported above were obtained using pure dencichine standard, as it is not possible to have a blank *P. notoginseng* matrix without the presence of dencichine. This is an intrinsic limitation in the analysis of natural components present in natural products and the method used here for validation is a pragmatic approach to the validation of such samples. To investigate the accuracy, recovery studies were performed by spiking *P. notoginseng* samples with different amounts of dencichine. The recoveries of dencichine at the three spiked

concentration levels ranged from 94.9 to 98.9% (n=3 for each concentration). The precision for the spiked samples was found to be 4.6-9.1%.

5.3.5 Quantification of dencichine

The concentrations (% w/w) of dencichine in all the samples are presented in Table 5.1. The eight raw *P. notoginseng* samples were found to have dencichine concentrations ranging from 0.134 to 0.293% w/w. Thus, a typical piece of root will contain 14 to 47 mg of dencichine, depending on the size of the roots. According to the Chinese Pharmacopoeia [The State Pharmacopoeia Commission of PR China, 2000], the recommended daily dose of raw *P. notoginseng* is about 3 to 9 g, so this will correspond to about 4 to 26 mg of dencichine. These variations in concentration were not unexpected as the roots were obtained from different sources. The quality and chemical contents of medicinal herbs may vary greatly due to many factors, e.g. species variation, geographical source, and methods used in cultivation, harvest, storage and processing. In this study, the sample obtained from Wenshan (China) (sample 7) was found to contain the highest dencichine amount. The findings obtained in this study are also consistent with previous reports. Raw *P. notoginseng* samples were reported to contain 0.316-0.382% w/w dencichine, using high-performance TLC technique [Zhang *et al*., 1990]. Long *et al.* [1996] studied dencichine from *Panax* species using anion exchange column chromatography, and reported a concentration of 0.16% w/w in the main roots. Dong *et al.* [2003] showed the regional and seasonal variations in dencichine concentration in raw *P. notoginseng* sample, from 0.325 to 0.615% w/w, with roots from Wenshan having the highest concentrations of dencichine.

Table 5.1. Concentrations (% w/w, n=3) of dencichine in raw *P. notoginseng*, steamed *P. notoginseng* (2, 6 and 9 h), *P. ginseng* and *P. quinquefolium* samples

Sample	Concentration of dencichine (% w/w)
Raw *P. notoginseng* sample 1	0.2361
Raw *P. notoginseng* sample 2	0.1814
Raw *P. notoginseng* sample 3	0.1927
Raw *P. notoginseng* sample 4	0.1739
Raw *P. notoginseng* sample 5	0.1827
Raw *P. notoginseng* sample 6	0.1337
Raw *P. notoginseng* sample 7	0.2935
Raw *P. notoginseng* sample 8	0.1625
Steamed (2h) *P. notoginseng*	0.0283
Steamed (6h) *P. notoginseng*	0.0004
Steamed (9h) *P. notoginseng*	0.0003
P. ginseng sample 1	0.0251
P. ginseng sample 2	0.0330
P. ginseng sample 3	0.0212
P. quinquefolium sample 1	0.0180
P. quinquefolium sample 2	0.0243
P. quinquefolium sample 3	0.0192
Korean red ginseng	0.0047

In this work, to determine the effects of steaming on *P. notoginseng* samples, a powdered raw sample was steamed for different durations and analysed. It was found that when the duration of steaming increased from 2 h to 9 h, the concentration decreased from 0.0283% w/w to a negligible amount of 0.0003% w/w. A Student's-t test also showed that the values were significantly decreased ($p<0.05$) compared to the corresponding raw *P. notoginseng* sample (before steaming). Thus, the greater the degree of steaming, the greater is the differences in the concentrations of dencichine in raw and steamed samples. The effects of cooking, roasting, autoclaving and fermentation on the concentration of dencichine (reported as β-ODAP) in *Lathyrus sativus* were previously studied, and a similar reduction in concentration was reported [Akalu *et al*., 1998]. A higher dencichine concentration in raw *P. notoginseng* may indicate that it has better haemostatic activity than the steamed samples and this is desirable for therapeutic uses.

Low doses of dencichine [Zhao and Wang, 1986], e.g. from the normal consumption of *P. notoginseng*, is unlikely to cause neurotoxicity. The dose of dencichine causing neurotoxicity was approximately more than 500 mg/kg in chicks (20-60 mg of dencichine was given to 30-40 g chicks) and the lethal dose (LD_{50}) was reported to be 1043 mg/kg. Whereas, 0.1 to 1 mg of dencichine was tested for haemostasis in 15-20 g mice and this was equivalent to ~5-50 mg/kg [Zhao and Wang, 1986]. In humans, it was reported that daily chronic consumption of 300 g of grass pea seeds (or when a diet consisting of one-third to one-half of peas is consumed) for more than 3 months may lead to neurotoxicity and this would be equivalent to 5-125 mg/kg/day (assuming that the concentration of dencichine in grass pea seeds ranges from 0.1-2.5% w/w and the average weight of man is 60 kg) [Akalu *et al*., 1998]. However, to date, there are no human data on the use of dencichine for haemostasis.

The present HILIC- MS/MS method was also applied to the analysis of 11 pairs of raw and steamed *P. notoginseng* CPMs, and the results are shown in Table 5.2. Except for two pairs, most of the raw CPM samples were found to contain 0.093-0.212% w/w of dencichine and the corresponding steamed CPM samples contained 0.015-0.085% w/w. For pair 10, the dencichine concentrations of raw and steamed samples were comparable, showing that the steamed sample may not have been steamed sufficiently or was not subjected to heat processing. For pair 11, the raw sample was found to contain a low concentration (0.013% w/w) of dencichine, comparable to that of most of the steamed samples. This implies that the raw sample may in fact have been subjected to some steaming or heat treatment during processing. These results correlated with the results from Chapter 3 on the comparison of saponin concentrations and chromatographic profiles in raw and steamed *P. notoginseng*. The pairs of products (pairs 10 and 11) were also found to have chromatograms not characteristic of their 'raw' and 'steamed' labels. Except for one pair (pair 10), all the steamed samples contained significantly ($p<0.05$, Student's-t test) lower amounts (2.5 to 12 times lower) of dencichine compared to the corresponding raw samples. From these results of dencichine concentrations, the varying degrees in which the CPMs are processed and its haemostatic activity may also be predicted.

As for the other *Panax* species, three sources of *P. ginseng* roots were found to have lower concentrations (0.021-0.033% w/w) of dencichine, while the concentrations in the three sources of *P. quinquefolium* roots were 0.018-0.024% w/w (Table 5.1). Therefore, these two species have a much lower content of dencichine (~ 4 to 16 times lower) compared to raw *P. notoginseng* roots. This may be one of the reasons why raw *P. notoginseng* has been widely used as a haemostatic agent, but haemostatic activity is not the main indication for *P. ginseng* and *P. quinquefolium*. A

Table 5.2. Concentrations (% w/w, n=3) of dencichine in 11 pairs of raw and steamed *P. notoginseng* CPMs

Pair no.	Product name	Brand	Concentration of dencichine (%w/w)
1	Pure Raw Pseudoginseng Powder	Meihua	0.2052
	Pure Steamed Pseudoginseng Powder	Meihua	0.0171
2	Raw Tienchi Ginseng Tablet	Meihua	0.1297
	Steamed Tienchi Ginseng Tablet	Meihua	0.0195
3	Yunnan Tienchi Powder (Raw)	Nature's Green	0.2117
	Yunnan Tienchi Powder (Steamed)	Nature's Green	0.0852
4	Yunnan Tienchi Tablets (Raw)	Nature's Green	0.1250
	Yunnan Tienchi Tablets (Steamed)	Nature's Green	0.0509
5	Tienchi Powder (Raw)	Yunfeng	0.2110
	Tienchi Powder (Steamed)	Yunfeng	0.0221
6	Tienchi Tablets (Raw)	Yunfeng	0.1344
	Tienchi Tablets (Steamed)	Yunfeng	0.0151
7	Tienchi Powder (Raw)	Camellia	0.1788
	Tienchi Powder (Steamed)	Camellia	0.0418
8	Tienchi Tablets (Raw)	Camellia	0.0926
	Tienchi Tablets (Steamed)	Camellia	0.0245
9	Tienchi Tablet (Raw)	Yulin	0.1353
	Tienchi Tablet (Steamed)	Yulin	0.0450
10	Yunnan Tian Qi Powder (Raw)	Kiat Ling	0.1117
	Yunnan Tian Qi Powder (Steamed)	Kiat Ling	0.1250
11	Chinese Yunnan Tien Chi Tablet (Raw)	Luen Shing	0.0128
	Chinese Yunnan Tien Chi Tablet (Steamed)	Luen Shing	0.0038

previous study [Kuo *et al*., 2003] also reported that a 3-year-old *P. ginseng* root contained 0.0596% w/w of dencichine. This is slightly higher than the results of this study. Differences may be due to the age, quality and source of the ginseng roots. In this study, a Korean red ginseng, a type of steamed *P. ginseng*, was analysed and found to contain 0.005% w/w of dencichine, a lower concentration than that in Chinese ginseng (white). This result further demonstrated that dencichine concentration is decreased on steaming. Long *et al.* [1996] also mentioned that red ginseng has lower dencichine concentration than white ginseng, but the actual quantities were not reported. Similarly, by using an amino acid analyser, Zheng and Li [1990] found that red ginseng contained less (0.2605% w/w) dencichine compared to white ginseng (0.4906% w/w).

Thermal isomerisation of dencichine to form α-*N*-oxalyl-L-α,β-diaminopropionic acid (α-ODAP) (Figure 5.1B) can occur. α-ODAP is non-neurotoxic unlike β-ODAP. Intramolecular rearrangements involving cyclic intermediates such as diketopiperazine types were proposed [De Bruyn *et al*., 1994]. The isomerisation is an equilibrium process whereby a particular equilibrium position is reached depending on the conditions used. Studies [Abegaz *et al*.; 1993, Belay *et al*., 1997b; Zhao *et al*., 1999b] showed that this occurred in acidic conditions and when the temperatures were in the range of 55-70˚C. Derivatisation methods that required some heating may promote isomerisation. In the preliminary studies, derivatisation by adding butanolic HCl, heating at 60˚C and evaporating the solvents with a stream of air, to form mono- or di-esters has been attempted. However, the results were not reproducible and it may have caused isomerisation of dencichine. Therefore, the present method of analysing underivatised dencichine is advantageous to prevent any inaccurate results obtained due to isomerisation or degradation. Besides isomerisation,

it has been reported that a dencichine solution can also be hydrolysed directly at high temperatures, in acidic or basic medium, to give α,β-diaminopropionic acid (DAP) (Figure 5.1C) [Akalu *et al*., 1998; Abegaz *et al*., 1993; De Bruyn *et al*., 1994; De Bruyn *et al*., 1993]. This is also the principle behind the analysis of dencichine using the o-phthalaldehyde (OPT) method, in which dencichine is hydrolysed into DAP prior to reaction with OPT [Rao, 1978; Briggs *et al*., 1983]. As high temperatures (>100˚C) and long durations are used, the change in dencichine concentration on steaming is likely due to thermal hydrolysis.

In the present work, this hydrolysis was investigated experimentally by HILIC/MS/MS monitoring of DAP; MRM transitions of *m/z* 105 → 87.9 and 105 → 69.8 were used. The MS/MS instrumental parameters used were similar to those used for dencichine, except for the optimal collision energy of 10 eV. Under the same LC conditions DAP eluted at 1.40 min, while dencichine eluted at 1.15 min (Figure 5.5). Dencichine standard also showed a peak for the MRM transition *m/z* 105 → 87.9, but it appeared at the same retention time (~1.15 min) as that for the transition *m/z* 177 → 116, i.e., the protonated DAP determined at 1.15 min is one of the MS product ions of dencichine, and did not arise from pre-ionisation decomposition of the dencichine standard. Dencichine standard stock solution (1000 ng/μl) was subjected to a temperature of 90°C for 14 h. It was then diluted and analysed by HILIC/MS/MS, showing both dencichine and DAP peaks after heating. Raw and steamed root samples were also analysed for DAP, and it was found that the DAP peak was higher in the steamed than in the raw samples. Therefore, these findings showed that dencichine was hydrolysed to DAP upon steaming, thus accounting for the differences in its concentration in raw and steamed *P. notoginseng* samples.

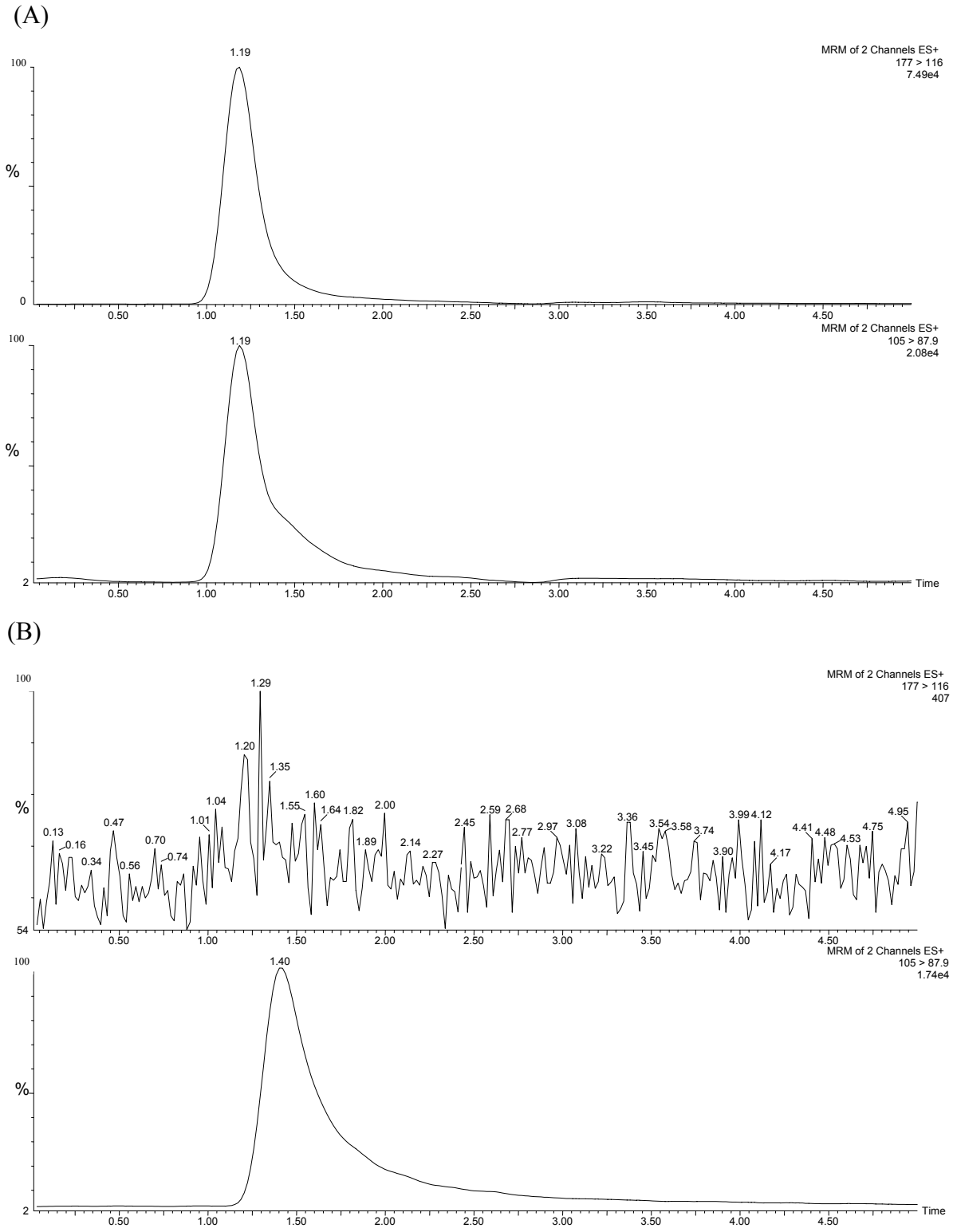


Figure 5.5. LC/MRM chromatograms of (A) dencichine standard showing ions detected for each of the two MRM transitions, *m/z* 177 → 116 (top) and *m/z* 105 → 87.9 (bottom); and (B) diaminopropionic acid (DAP), showing the absence of ions detected for MRM transition *m/z* 177 → 116 (top) and presence of ions detected for the transition *m/z* 105 → 87.9 (bottom).

5.4 Conclusion

In this chapter, a novel HILIC/ESI-MS/MS method has been successfully developed for the analysis of underivatised dencichine in *Panax* species. Compared to other conventional methods, this technique is rapid (5 min), selective, sensitive and eliminates the need for sample derivatisation. The method has been validated for linearity, accuracy, intra- and interday precision, LOD and LOQ. Results showed that steamed *P. notoginseng* samples contained less dencichine when compared to the raw samples, due to the hydrolysis of dencichine to diaminopropionic acid at high temperatures. *P. ginseng* and *P. quinquefolium* were also found to have lower dencichine concentration than *P. notoginseng*. A higher concentration in raw *P. notoginseng* root may indicate that it has a better haemostatic activity. The method developed will be useful for the determination of dencichine for the quality control of *Panax* species, as well as other food crops that may contain this component.

CHAPTER 6

PLATELET AGGREGATION AND BLOOD COAGULATION INHIBITORY ACTIVITIES OF *PANAX NOTOGINSENG*, ITS RELATED SPECIES AND ITS CHEMICAL COMPONENTS

6.1 Introduction

Platelets play a central role in the haemostatic process and are similarly involved in the pathological process—thrombosis. They are small discoid cells derived from megakaryocytes in the bone marrow and circulate in the blood. Under normal conditions, they neither adhere to each other nor other circulating endothelial cells. When the blood vessels are damaged, subendothelial cells are exposed and platelets adhere to the disrupted surface. This adhesion is mediated by collagen and von Willebrand factor (vWF) on the subendothelial surface. After adhesion, platelet activation occurs, a process that is stimulated by the interaction of several agonists such as ADP, thrombin, epinephrine, with their specific receptors on the platelet surface. Phospholipase A_2 enzyme is also activated to produce a variety of prostaglandins and thromboxane. The adherent and activated platelets will subsequently secrete or release various biologically active factors such as ADP, serotonin, fibrinogen from their platelet granule content. Release of factors from granules, which together with thromboxane A_2, can promote further platelet activation, recruit other platelets to the site of injury and promote further aggregation to form the platelet plug. The aggregation (defined as the adherence of one platelet to another) is mediated by fibrinogen or vWF, which connect the platelets by bridging complexes of

glycoprotein GPIIb/IIIa on adjacent platelets [Freson *et al*., 2006]. Therefore, there are a variety of platelet agonists and the binding of agonists to specific platelet membrane receptors is important to induce adhesion, activation and aggregation processes. Inhibition of any of these factors or steps may result in the final inhibition of platelet aggregation, i.e. antiplatelet effects. For example, collagen is found in the connective tissue of blood vasculature and is considered to be one of the initial stimulating factors that the platelet encounters following vascular trauma. The study of the platelet response to collagen is important in many platelet aggregation studies.

The activation of platelets with the exposure of negatively charged phospholipids facilitates the assembly of coagulation factors onto the surface and activation of the coagulation cascade to generate thrombin and subsequent fibrin deposition to stabilise the initial primary platelet plug. This secondary haemostasis process is also needed for thrombus growth, development and stability. The coagulation cascade is basically divided into the intrinsic, extrinsic and common pathways. Intrinsic pathway is activated by surface contact with negatively charged phospholipids (provided by activated platelets or platelet activation factors) and involves the sequential activation of various factors, Factors XII, XI, IX. Factor X can then be activated by Factors IXa and VIII. Extrinsic pathway is initiated by the release of tissue thromboplastin which is exposed during vascular injury. Factor VII forms a complex with tissue thromboplastin and calcium. This complex rapidly activates Factor X to Xa which is needed to activate the final pathway. This common pathway is initiated by both the activated intrinsic and extrinsic pathways which produces the activated Factor X. Factor Xa, in the presence of Factor V, phospholipid surface and calcium, catalyses the conversion of prothrombin (Factor II) to thrombin (Factor IIa) which is needed to convert fibrinogen (Factor I) to fibrin (clot). To ensure the

physiological balance of haemostasis and avoid massive clot formation, the coagulation process is controlled by several mechanisms such as natural inhibitors in plasma (e.g. antithrombin III, protein C) and the fibrinolysis system. The fibrinolytic pathway involves activation of plasminogen to plasmin, which is needed to degrade or split fibrinogen and fibrin into fragments (Fibrin degradation products, FDP). A disruption of this fine balance of haemostasis and fibrinolytic system can result in bleeding or further thrombosis. The whole coagulation cascade involves a complex number of coagulation factors, which are mostly proteins circulating in the plasma and are generally synthesised in the liver. A deficiency in any coagulation factors or the presence of any circulating or exogenous inhibitors of the factors will thereby inhibit the coagulation cascade and prevent the formation of a clot or thrombus.

As discussed in Section 1.2.1, inhibitions of the platelet function and coagulation pathways represent a promising approach for the prevention of thrombosis in pathological conditions. A thrombus is a source of thromboembolic complications of cardiovascular diseases, and hence antiplatelet and anticoagulation agents are used in such diseases. In view of the importance of antithrombotic therapy in cardiovascular diseases and the growing number of drugs obtained from botanical sources, medicinal herbs and their phytochemicals that prevent thrombosis by inhibition of either platelet functions or coagulation pathways are, therefore, of special therapeutic interest.

According to the Chinese Pharmacopoeia, raw *P. notoginseng* has been traditionally used as a haemostatic to arrest internal and external bleeding, reduce swelling and pain, as well as to disperse blood clots, eliminate blood stasis and promote blood circulation [The State Pharmacopoeia Commission of PR China, 2000]. Interestingly, promoting circulation and enhancing haemostasis seems to be of

opposite effects scientifically. Studies also showed that raw *P. notoginseng* has other useful pharmacological activities on cardiovascular related diseases such as lower blood cholesterol, reduce blood pressure, treatment of coronary heart diseases, cardiac infarction, angina pectoris [State Administration of Traditional Chinese Medicine, 1996; Tang and Eisenbrand, 1992]. However, currently, studies on the antithrombotic activities of *P. notoginseng* are limited. Moreover, the studies on *P. ginseng* cannot be directly postulated to *P. notoginseng*. In previous chapters, it was found that the chemical components in raw and steamed *P. notoginseng* were different and the steamed form contained several unique saponins that were produced during processing. Although the chemical components are different, to date, there are no scientific studies to show if they have different biological activities. Furthermore, several of their unique components have not been tested for antithrombotic activities.

Therefore, this chapter aims to determine the effects of different forms of *P. notoginseng* and its related species, on blood platelet aggregation and coagulation (*in vitro* and *ex vivo*). In addition, *in vivo* bleeding time assays will be carried out to investigate their haemostatic effects in the *in vivo* system. The activities of the chemical components of *P. notoginseng* on blood coagulation and platelet aggregation (*in vitro*) will also be investigated, so as to understand the components that are contributing to the activities and to identify potential leads for antithrombotic drugs.

6.2 *In vitro* platelet aggregation and blood coagulation studies

6.2.1 Experimental

6.2.1.1 Materials

Raw *P. notoginseng* roots were purchased from different sources in Singapore (Samples 1-3) and China (Samples 4-6), as mentioned in Section 3.1.2.1. Several raw *P. notoginseng* roots were also collected from Wenshan (Yunnan, China) (Sample 7, WS-7, 4, 5, 11), while reference authenticated raw *P. notoginseng* (Sample 8) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Four pairs of raw and steamed *P. notoginseng* CPMs (CPM pairs 1, 9, 10 and 11) were bought (Table 3.1, Section 3.1.2.1). *P. ginseng*, *P. quinquefolium*, Korean red ginseng and reference authenticated red ginseng roots were also obtained, as described in Section 3.1.2.1.

Dencichine, diaminopropionic acid (Section 5.2.1), ginsenosides Rg1, Re, Rb1, Rc, Rd and notoginsenoside R1 (Section 3.1.2.1) were purchased. 20S- and 20R-ginsenoside Rg3 from steamed *P. notoginseng* were obtained as a gift (Section 4.2.1), while 20S-ginsenoside Rh1, 20R-ginsenoside Rh1, ginsenoside Rk3, Rh4, Rk1 and Rg5 were isolated in the laboratory as described in Chapter 4 (purity >95%). Water, hexane and butanol fractions of steamed *P. notoginseng* were also obtained by the procedures described in Chapter 4.

Four ml sterile polypropylene tubes (Tapval®) with 0.106 M (3.8%) citrate (1 part citrate: 9 parts blood) obtained from J.P. Selecta, S.A. (Abrera, Spain), were used to collect the blood from rabbits and rats. Ketamine (Ketapex, 100 mg/ml) and xylazine hydrochloride (Ilium Xylazil-20, 20 mg/ml) was obtained from Apex Laboratories Pty Ltd (NSW, Australia) and Troy Laboratories Pty Ltd (NSW,

Australia) respectively. Carboxymethylcellulose (CMC) was from BDH Ltd (Poole, UK).

For platelet aggregation experiments, collagen (1 mg/ml) from equine tendons, suspended in isotonic glucose solution of pH 2.7 was purchased from Chrono-Log Corporation (Havertown, PA, USA). Phosphate buffered saline (PBS, pH 7.4) containing 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4 was prepared. Aspirin USP grade was purchased from Sigma-Aldrich (St. Louis, MO, USA).

For blood coagulation experiments, the following reagents were obtained from Dade Behring Marburg GmbH (Marburg, Germany): Ci-Trol level 1 (lyophilised human plasma with stabilisers and buffers), Thromborel S (Human placental thromboplastin containing calcium chloride, stabilisers and preservatives), Actin FSL (Liquid purified soy and rabbit brain phosphatides in 0.1 mM ellagic acid activator with added buffer, stabilisers and preservatives), Calcium chloride (25 mM), Thromboclotin (Bovine thrombin, approximately 2.5 NIH units/ml), Batroxobin (an enzyme from the venom of *Bothrops atrox*), Thrombin (Bovine thrombin, approximately 100 NIH units/ml), Owren's Veronal buffer (pH 7.35), Standard human plasma (Pooled citrated human plasma stabilised with 12 g/L hepes buffer and lyophilised), Control plasma N (normal values, pooled citrated human plasma stabilised with 12 g/L hepes buffer and lyophilised), Control plasma P (pathological values, pooled citrated human plasma adjusted to defined factor concentrations, stabilised with 12 g/L hepes buffer and lyophilised). Heparin sodium salt (180 USP units/mg) from porcine intestinal mucosa was purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.2.1.2 Sample preparation

The roots were ground into powder using a laboratory blender. 5 g of each powdered sample were accurately weighed and extracted with 50 ml of 70% methanol by ultrasonication for 20 min. The extracts were filtered and the ultrasonication process was repeated for two additional times. The combined filtrate was evaporated to dryness. The residue was weighed and dissolved in appropriate volumes of PBS. The mixture was then filtered through a 0.45 μm nylon filter membrane and diluted to appropriate concentrations with PBS. Samples of a powdered raw *P. notoginseng* root were steamed at 120°C for 2, 6 and 9 h (Section 3.1.2.3), and extracted using the same procedure as described above.

6.2.1.3 Animals for blood collection

Male New Zealand Whites rabbits (2.5-4 kg) were purchased from Laboratory Animal Centre, Singapore, and housed at the Animal Holding Unit of National University of Singapore. The care of the animals and collection of blood from rabbits were carried out in accordance to internationally accepted guidelines on laboratory animal use and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore. They were maintained healthy in an air-conditioned room at a temperature of 22 ± 1°C and a humidity of 62 ± 2%. The animals were allowed unrestricted access to food (commercial pellet diet for rabbits) and water. The rabbits were acclimatised for at least a week before any experimentation.

6.2.1.4 Blood collection

Fresh whole blood was collected from the ear vein of healthy male rabbits. The rabbit was sedated with a mixture of ketamine (20 mg/kg) and xylazine (4 mg/kg), given intramuscularly. Blood was dripped from a 22G needle directly into the 4 ml-citrated tubes and the total volume collected during each session was not more than 0.67% (if rested for at least 14 days) or 1% of its body weight (if rested for at least 21 days). To prevent coagulation, the tubes were gently inverted to ensure a good mix of citrate and blood. The blood was immediately used after collection. The rabbits were rested for at least 14 to 21 days before the next blood collection to ensure sufficient recovery of the blood platelets and blood cells.

6.2.1.5 *In vitro* platelet aggregation assays

Platelet aggregation experiments were carried out using Chrono-Log model 570VS four-channels whole blood aggregometer, electrodes and cuvettes obtained from Chrono-Log Corporation (Havertown, PA, USA). The whole blood aggregation assays were determined by the electrical impedance method [Cardinal and Flower, 1980]. 450 μl of pre-warmed (37°C) whole blood sample from rabbits was transferred into a siliconised plastic cuvette and diluted with an equal volume (1:1) of pre-warmed (37°C) PBS (pH 7.4). The cuvette was placed in the reaction well of the aggregometer which was pre-warmed to 37°C. The diluted blood sample inside the cuvette was stirred with a polytetrafluoroethylene-coated magnetic stir bar at a constant speed of 1000 rpm throughout the experiment. The diluted blood sample was equilibrated for 2 min and once a stable baseline reading was achieved, 20 μl of test sample solution (or control) was added into it. The blood and test sample were incubated for 2 min, then 2 μl of collagen (to give a final concentration of 2 μg/ml)

was added to initiate the aggregation reaction. The platelet aggregation was monitored for 5 min by electrical impedance. The results were processed and analysed using Aggro/link version 4.75 software. The impedance value (in ohms) was determined from the impedance graph, starting from the lowest point after the start of the reaction to the time point exactly at the end of the 5 min time interval. The whole blood samples were used within 4 h after blood collection and controls were analysed at various intervals in between the test samples to ensure adequate activities of the platelets.

6.2.1.6 *In vitro* blood coagulation assays

Blood coagulation experiments were performed using Sysmex CA-530 blood coagulation analyser obtained from Sysmex Corporation (Kobe, Japan). The assays carried out include prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), reptilase time and fibrinogen determination test.

Preparation of plasma sample

The lyophilised pooled human plasma (Ci-Trol level 1) was reconstituted with 1 ml of distilled water and left to stand for at least 15 min. It was used within 8 h after reconstitution. The plasma mixtures were prepared by mixing 100 μl of plasma with 50 μl of test sample (2:1 dilution) in a sample cup before performing the following assays. For the individual compounds, 3 mM of the samples were prepared to give a concentration of 1 mM in the sample cup after the addition of plasma.

Prothrombin time (PT)

Thromborel S was reconstituted with distilled water to the labelled volume. It was warmed and maintained at 37°C for at least 30 min before use. 50 µl of the plasma mixture was pipetted into a reaction tube and incubated at 37°C for 180 s. After incubation for 180 s, 100 µl of Thromborel S reagent was added and the tube was vortexed to ensure good mixing of the reagents. The reaction was monitored for 120 s. Detection was based on light scattering principle, whereby the reaction tube was irradiated with light at 660 nm, and the change in scattered light due to turbidity change during conversion of fibrinogen to fibrin was detected. The time taken to reach 50% change in scattered light was regarded as the clotting time.

Activated partial thromboplastin time (APTT)

50 µl of the plasma mixture was pipetted into a reaction tube and incubated at 37°C for 60 s. After incubating for 60 s, 50 µl of Actin FSL reagent was added and further incubated for 180 s. 50 µl of calcium chloride was then added and the reaction was monitored for 190 s. Detection was based on the same principle as described above.

Thrombin time (TT)

100 µl of the plasma mixture was pipetted into a reaction tube and incubated at 37°C for 60 sec. 100 µl of Thromboclotin reagent was then added and the reaction was monitored for 100 sec.

Reptilase time

50 µl of the plasma mixture was pipetted into a reaction tube and incubated at 37°C for 60 s. 100 µl of Batroxobin reagent was then added and the reaction was monitored for 100 s.

Fibrinogen determination test

A calibration curve was obtained with various concentrations of standard human plasma (100-500 mg/dl) diluted with Owren's Veronal Buffer (OVB). For the analysis of the fibrinogen, 10 µl of the plasma mixture was pipetted into a reaction tube and diluted with 90 µl of OVB. It was then incubated at 37°C for 180 s. 50 µl of Thrombin reagent was added and the reaction was monitored for 100 s.

6.2.1.7 Statistical analysis

The experimental results were expressed as mean ± SD. Statistical analyses were performed using Student's t test (for comparisons between two groups) and one-way analysis of variance (ANOVA) (for comparisons between three or more groups) with Tukey-Kramer post hoc test. Data were considered statistically significant when p values were <0.05. IC_{50} values (50% inhibitory concentration) were calculated using non-linear regression from Graphpad Prism version 3.00 for Windows (Graphpad Software Inc., San Diego, CA, USA).

6.2.2 Results and Discussion

6.2.2.1 *In vitro* platelet aggregation assays

In this study, platelet aggregation assays using whole blood was selected and used. Electrical impedance was selected as the measure of platelet aggregations. Figure 6.1 shows the typical changes in impedances with time for a typical control using PBS. After the addition of test samples or controls, a two min equilibration was found to be sufficient to gradually stabilise the baseline prior to the addition of collagen. This equilibration was needed to ensure that the blood, PBS and sample were thoroughly mixed, to allow the temperature to reach 37°C and to ensure that a monolayer of platelets was coated on the electrode. Collagen was then added to induce platelet aggregations and the impedance increased to a maximum. Figure 6.1 also shows the changes in impedances with time for aspirin. After the induction by collagen, the increase in impedance was low, as it is known that aspirin inhibits platelet aggregations by inhibiting the cyclooxygenase enzyme (Chapter 1). This method was capable of detecting antiplatelet activities of aspirin. It served as a positive control for comparisons in this study. The absolute degree of platelet aggregation was expressed as a percent inhibition (Y), which was calculated using the following equation:

$$Y\ (\%) = [(A-B)/A] \times 100$$

where, A = maximal impedance value (in ohms) of control

B = maximal impedance value (in ohms) of sample

The maximal impedance value of the control was regarded as 100% platelet aggregation and the impedance values of the samples were compared with the control within the same reaction time interval of 5 min. If the added sample inhibits the platelet aggregation process, the increase in impedance value will be slower and the

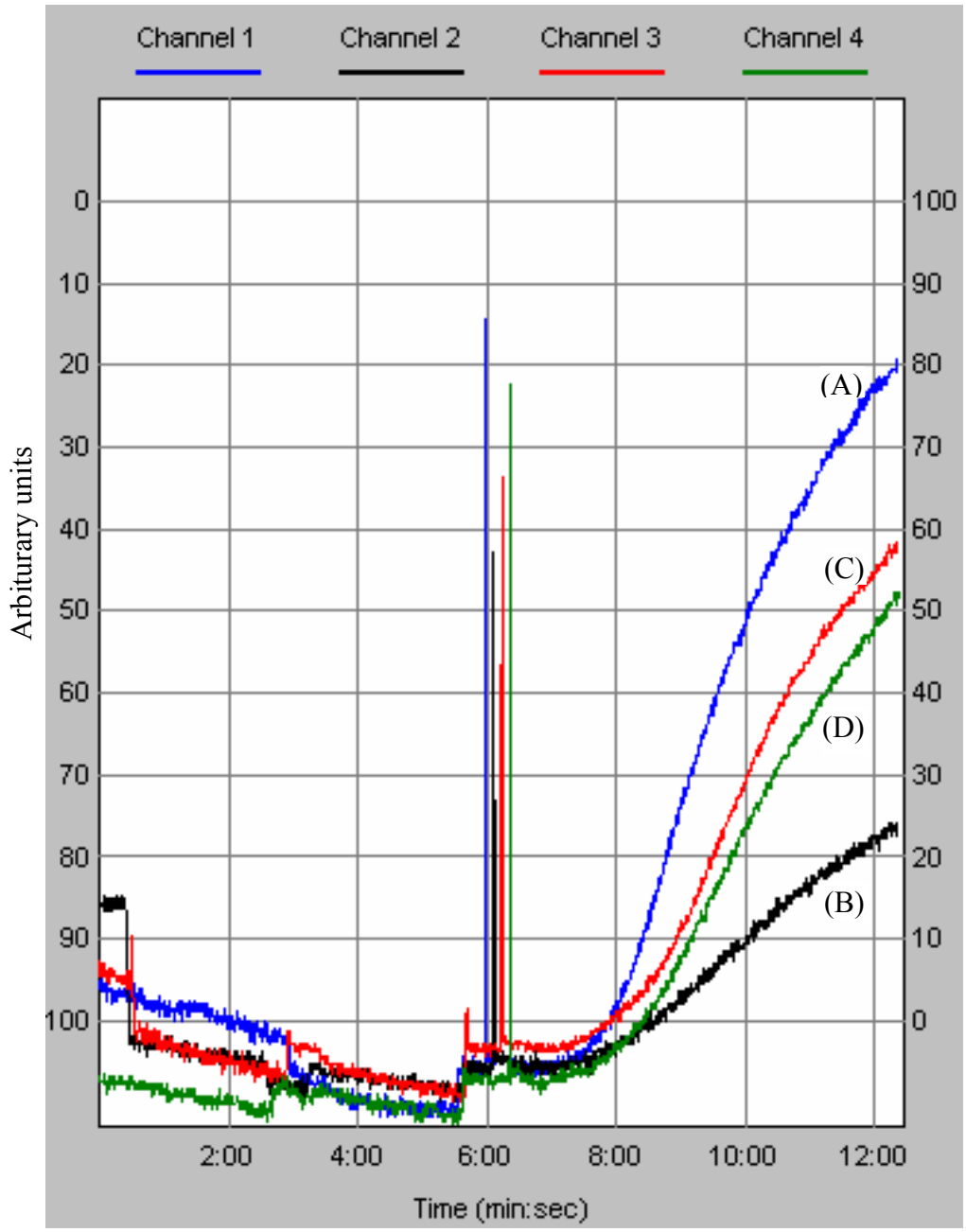


Figure 6.1. Graph showing the effects of (A) control (PBS), (B) aspirin, (C) raw and (D) steamed (2 h) *P. notoginseng* on the changes in electrical impedances in whole blood, using collagen to induce platelet aggregations.

final impedance value will be lower than that of the control. The higher the % inhibition of platelet aggregation, the greater will be its antiplatelet effects.

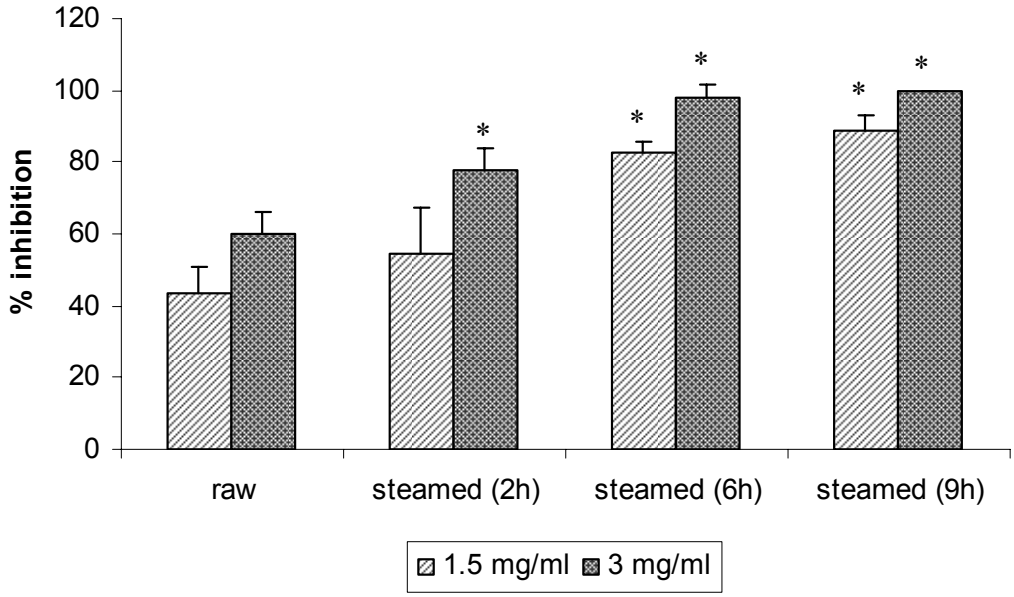
The basic principle [Cardinal and Flower, 1980] behind the whole blood platelet aggregometer is that the electrodes will become coated with a monolayer of platelets during the initial contact with blood. If no further interactions occur between the platelets and electrode, the conductance between the two electrodes will be kept constant. However, when platelet aggregation occurs, there will be a gradual aggregation of platelets onto the platelet monolayer coated on the electrodes. This impairs conduction between the two electrodes and the increased impedance can be recorded and measured. This use of whole blood is definitely more reflective of the physiological conditions than platelet-rich plasma. Although the Born turbidometric optical aggregometer [Born, 1962a; Born, 1962b] was the first device used for determining platelet aggregation and it was widely used in research, but its main limitation is that it functions only with translucent cell suspensions such as plasma. The other components of blood such as the leucocytes can generate anti-aggregating agents such as prostacyclin [Cardinal and Flower, 1980], so the determination of activities using whole blood will be different from that in platelet-rich plasma. Furthermore, during the separation of blood into platelet-rich plasma, some heavier platelets may sediment together with the red cells. Preparation of platelet-rich plasma will also increase the sample preparation time and labile modulators such as thromboxane A_2 and prostacyclin may decay. From the above, it is evident that the use of whole blood with electrical impedance detection has important advantages over platelet-rich plasma with turbidometric detection.

In vitro platelet aggregation assays of extracts of _P. notoginseng_ and its related species

When raw and steamed *P. notoginseng* samples were added, the increase in impedance was slower and the maximum was lower as compared to control (Figure 6.1). As shown in Figure 6.2, raw *P. notoginseng* samples (sample 2) showed 59.8% inhibition (3 mg/ml) of platelet aggregation and the inhibition was dose dependent. The values were close to that obtained for the same concentrations of raw *P. notoginseng* reference standard (51.7%). Raw *P. notoginseng* root samples from the same GAP farm and from different sources (Section 6.2.1.1) were found to result in % inhibition ranging from 53.4% to 57.8%. The slight variations in their chemical contents (as discussed in Chapter 3) did not significantly change their platelet inhibitory activities. Thus, the components present predominantly in raw samples do not seem to significantly change their overall platelet aggregation activities.

On the other hand, steamed *P. notoginseng* samples were found to have a significantly higher platelet inhibition activity as compared to the raw samples at both concentrations, as shown in Figure 6.2. The percentage inhibition increased with increasing duration of steaming. For the raw sample, the IC_{50} was 3 mg/ml, while the value was 1.5 mg/ml for steamed (2 h) samples and less than 1.5 mg/ml for samples steamed for more than 2 h. Therefore, steamed *P. notoginseng* has potentially better antiplatelet effects. In Chapters 3, 4 and 5, it was shown that the components were changed according to the extent of steaming. Notoginsenoside R1, ginsenosides Rg1, Re, Rb1 and Rd were decreased, while 20S and 20R-ginsenoside Rh1, 20S and 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5 were increasingly formed with increasing extent of steaming (Figure 3.6 and 3.7). Therefore, these changes in chemical compositions may have resulted in an increased in activities and the new

components formed after processing may have good inhibitory effects. Four pairs of raw and steamed *P. notoginseng* CPM products, which have been studied in Chapter 3, were also investigated for their activities to see whether there was any relationship between their differences in chemical profiles and their activities. A pair of raw and steamed products, which showed differences in chemical analysis, was found to have 57.4% and 90.5% inhibition respectively and this was significantly different. Whereas, three pairs of raw and steamed products, which have similar chemical profiles, showed no significant differences in their activities between raw and steamed products. Therefore, this provided clear evidence that the inhibition of platelet aggregation was closely related to their chemical composition and the extent of steaming.



*: $p<0.05$ compared to raw *P. notoginseng*, one-way ANOVA

Figure 6.2. Platelet inhibitory effects of raw and steamed *P. notoginseng in vitro* (n≥3).

Among the three *Panax* species, *P. ginseng* and *P. quinquefolium* were found to have lower inhibitory activities ($p<0.05$) of 46.2% and 37.7% respectively (Table 6.1), as compared to raw *P. notoginseng* samples at the same final concentration of 3 mg/ml. Therefore, *P. notoginseng* may have the highest antiplatelet action compared to its closely related *Panax* species. Previous studies have been focused on *P. ginseng* and its components, so *P. quinquefolium* have not been studied for antiplatelet activities. Korean red ginseng and red ginseng reference standard were actually *P. ginseng* that have been heat or steamed-treated. Korean red ginseng was previously investigated and found to effectively inhibit *in vitro* platelet aggregations [Yun *et al*., 2001b] with an IC_{50} of 0.32 mg/ml. In this work, the inhibitory activities of Korean red ginseng (50.7%) and red ginseng reference standard (52.6%), were found to be slightly higher than the Chinese (white) ginseng. Therefore, this was consistent with the conclusion obtained for raw and steamed *P. notoginseng*, i.e. steamed form showed greater platelet inhibition. However, unlike the large differences between raw and steamed *P. notoginseng*, the differences between *P. ginseng* (Chinese) and red ginseng were not significant. From the chemical analysis, the extent in which red ginseng was changed by processing was not as large as the pairs of raw and steamed *P. notoginseng*.

Table 6.1. Platelet inhibitory effects of the various *Panax* species *in vitro* as compared to control (n≥3)

Sample	Final concentration (mg/ml)	% Inhibition
P. quinquefolium	3.0	37.7 ± 6.8
P. ginseng	3.0	46.2 ± 5.8
Korean red ginseng	3.0	50.7 ± 7.2
Red ginseng reference standard	3.0	52.6 ± 6.2
Raw *P. notoginseng*	3.0	59.8 ± 6.2
Steamed *P. notoginseng* (9 h)	3.0	100 ± 0.0

In vitro* platelet aggregation assays of different fractions of *P. notoginseng

The butanolic fraction of steamed *P. notoginseng* was found to inhibit platelet aggregation to the greatest extent, followed by the water and hexane fractions, and in a dose-dependent manner (Figure 6.3). Although the non-saponin or lipophilic fraction in *P. ginseng* was also reported to inhibit platelet aggregation *in vitro* [Park *et al*., 1995] and *in vivo* [Park *et al*., 1996], in this work, the majority of the antiplatelet components were found to be present in the butanolic fraction of *P. notoginseng*. Chemical analysis of the butanolic fraction using HPLC showed that it contained the majority of saponins. Therefore, the saponins were proposed to have good antiplatelet activities. This was supported by studies on *P. ginseng* which showed that the total ginsenosides inhibited platelet aggregation, inhibited release of platelet granule contents and inhibited morphological changes [Mo *et al*., 1989]. The 1-butanol fraction and diethyl ether fraction of *P. ginseng* were also reported to suppress the

ATP release reaction and inhibit aggregation, with the latter being more potent than the former [Kuo *et al*., 1990]. The *P. notoginseng* total saponins and panaxatriol saponins were found to inhibit platelet aggregation and adhesion [Ma and Xiao, 1998; Wang *et al*., 2004], increase the fluidity of platelet membranes *in vitro* [Su *et al*., 1996] and inhibit shear-induced platelet aggregation [Liao and Li, 1997]. Isolation of the various saponins was subsequently carried out in Chapter 4. These saponins were individually determined for antiplatelet effects in this chapter.

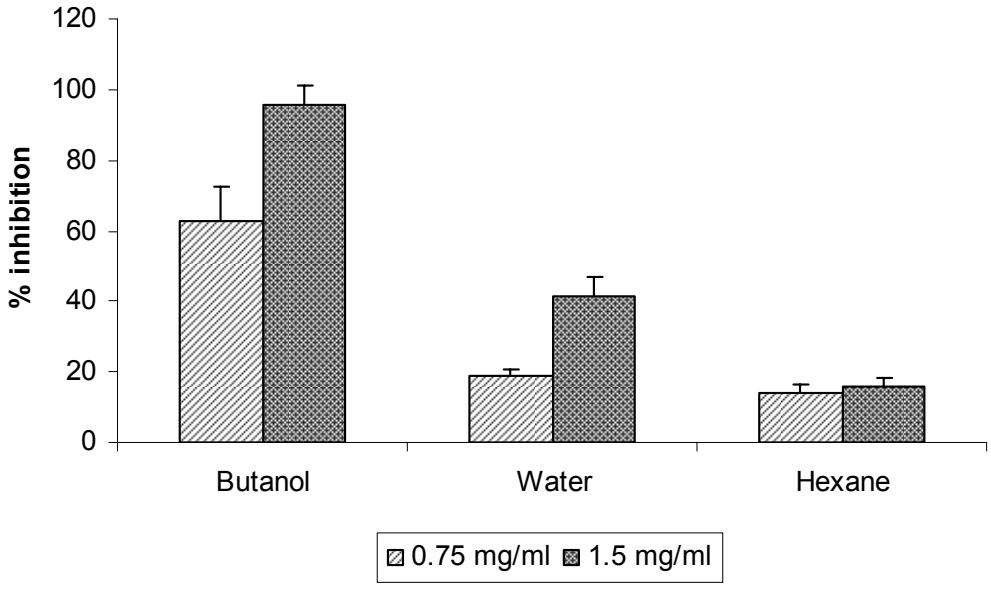


Figure 6.3. Platelet inhibitory effects of different fractions of steamed *P. notoginseng* *in vitro* (n≥3).

***In vitro* platelet aggregation assays of individual compounds**

Most of the individual saponins tested have moderate platelet inhibitory activities, ranging from 20% to 32% at 100 μM concentration, as shown in Table 6.2. Interestingly, 20R-ginsenoside Rh1 and 20R-ginsenoside Rg3 showed no antiplatelet activity unlike their corresponding 20S-epimer. On the other hand, ginsenoside Rh4 inhibited platelet aggregation up to 40%. The strongest activities came from ginsenoside Rg5 and 20S-ginsenoside Rg3 which have platelet inhibitory activities exceeding 45% at 100 μM. A previous study [Jung *et al*., 1998] also reported ginsenoside Rg3 (IC_{50} 49 μM) and Δ^{20}–ginsenoside Rg3 (IC_{50} 92 μM) to be a new class of platelet activating factor (PAF) antagonists which inhibits PAF binding to washed platelets. Currently, the antiplatelet effects of various ginsenosides varied greatly in different studies. Ginsenosides Ro, Rg1 and Rg2 from *P. ginseng* [Kuo *et al*., 1990] showed some inhibition of ATP release, but not inhibition of platelet aggregation. In another study [Matsuda *et al*., 1986a], ginsenosides Rg1, Rg2 and Rb1 inhibited platelet aggregations, but the inhibitions were weak with 10-25% at high concentrations of 1 mM. Ginsenoside Rg1 was shown to inhibit platelet aggregation and 5-HT release [Kimura *et al*., 1988]. Panaxynol [Kuo *et al*., 1990; Teng *et al*., 1989], a polyacetylene, isolated from the diethyl ether layer of *P. ginseng* was found to be an active antiplatelet component and its mechanism of action was chiefly due to the inhibition of thromboxane formation. Prior to this work, there are still several saponins, namely, notoginsenoside R1, 20RS-ginsenoside Rh1, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5, that have not been investigated for antiplatelet activities.

Table 6.2. Platelet inhibitory effects of dencichine, diaminopropionic acid and saponins *in vitro* as compared to PBS control (n≥3)

Sample	Final concentration (μM)	% Inhibition
Aspirin	100	26.7 ± 2.8
Notoginsenoside R1	100	22.5 ± 5.8
Ginsenoside Rg1	100	31.1 ± 6.1
Ginsenoside Re	100	24.2 ± 7.1
Ginsenoside Rb1	100	32.6 ± 4.7
Ginsenoside Rc	100	18.4 ± 3.3
Ginsenoside Rd	100	29.1 ± 6.4
20S-ginsenoside Rh1	100	24.7 ± 4.0
20R-ginsenoside Rh1	100	-
Ginsenoside Rk3	100	32.3 ± 7.5
Ginsenoside Rh4	100	40.6 ± 9.5
Ginsenoside Rk1	100	21.3 ± 6.2
Ginsenoside Rg5	100	48.2 ± 10.5
20S-ginsenoside Rg3	100	47.0 ± 12.5
20R-ginsenoside Rg3	100	-
Dencichine	100	6.2 ± 1.5
Diaminopropionic acid	100	21.2 ± 5.9

Unshaded: Chemical components present in raw and steamed *P. notoginseng* with the steamed form having lower quantities. Shaded: Chemical components present only in steamed *P. notoginseng*

The IC_{50} values of the two most potent saponins, ginsenosides Rg5 and 20S-Rg3, and aspirin were determined in this work to be 98 μM, 92 μM and 131 μM (Figure 6.4) respectively. The IC_{50} of aspirin was reported to be 139-160 μM [De La Cruz *et al*., 2002; Yun *et al*., 2001b] for collagen-induced platelet aggregations, which were closed to the results obtained in this study. Aspirin, which inhibits cyclooxygenase enzyme, is an antiplatelet drug widely used for prophylaxis of thrombosis. Another clinically used antiplatelet drug, ticlopidine, which prevents the activation of glycoprotein IIb/IIIa complex by platelet ADP, has a reported IC_{50} value of 513 μM [De La Cruz *et al*., 2002] for collagen-induced platelet aggregation. Therefore, these two ginsenosides, isolated from steamed *P. notoginseng*, demonstrated more potent platelet inhibitory effects than some of the clinically used drugs. The good antiplatelet effects of ginsenosides Rg5 and Rh4 have not been previously reported.

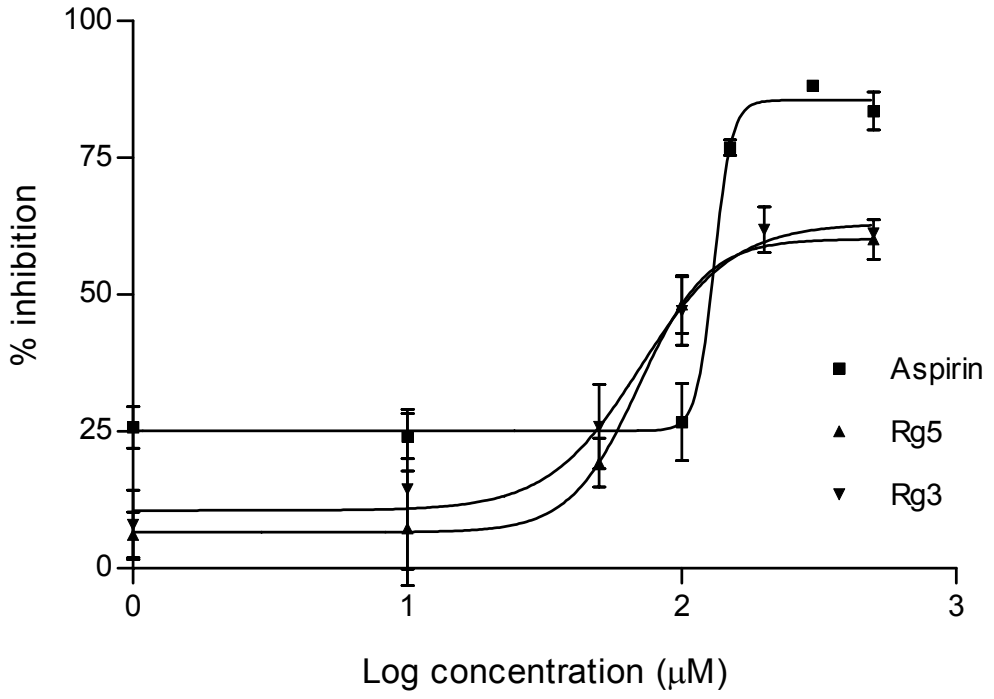


Figure 6.4. Dose response curves for aspirin, ginsenosides Rg3 and Rg5, showing the changes in percentage platelet inhibition with concentration (I: represents the standard deviations).

As discussed in Chapter 5, dencichine is a bioactive amino acid component present in raw *P. notoginseng* and it is reported to be responsible for *P. notoginseng's* haemostatic activity. However, it is not previously known if dencichine has any effects on platelet aggregation. The current results show that it has no effects on platelet aggregation. This was not unexpected as dencichine is known to have haemostatic activity (Table 6.2). The mechanism for haemostasis is still largely unknown. It has been reported to increase the platelet number in a study [Zhao and Wang, 1986], but it is not known if this is one of its main mechanism for its haemostatic effects. The content of dencichine was greatly reduced in steamed *P. notoginseng* samples (Chapter 5). Furthermore, diaminopropionic acid, a hydrolysed product from dencichine, was present in the steamed samples and it was found to be 3.4 times more active than dencichine, and with slightly less activity than aspirin at the same concentration. This may also be one of the reasons contributing to the higher inhibition activity seen in the steamed samples.

6.2.2.2 *In vitro* blood coagulation assays

The PT measures the activity of factors of the extrinsic pathway including Factors II (prothrombin), V, VII, X and I (fibrinogen). Complementarily, the APTT screens for abnormalities of the intrinsic coagulation pathway, including Factors I (fibrinogen), II (prothrombin), V, VIII, IX, X, XI, XII, prekallikrein, and high molecular weight kininogen (HMWK). Thrombin time (TT) measures the direct conversion of fibrinogen to fibrin in the common coagulation pathway by the addition of thrombin.

In vitro* blood coagulation assays of extracts of *P. notoginseng

The extracts of raw *P. notoginseng* were found to significantly prolong PT at the various concentrations used in the study and to significantly prolong APTT at higher concentration (6.7 mg/ml), as shown in Table 6.3. The changes were concentration dependent. Twelve different sources (Section 6.2.1.1) of raw *P. notoginseng* have also been investigated. The reference raw *P. notoginseng* standard was found to prolong PT (20.6 s) and APTT (44.4 s) to similar values as the other raw samples at the same concentration (5 mg/ml). The ranges of PT and APTT for various sources of roots were found to be 20.1 to 22.6 s and 42.3 to 50.8 s respectively. Despite some differences in the quantities of some chemical components (Chapter 3), various sources of raw *P. notoginseng* samples gave comparable results, suggesting that the variations in contents of notoginsenoside R1, ginsenosides Rg1, Re, Rb1, Rc and Rd did not significantly affect anticoagulant effects.

Extracts of steamed *P. notoginsen*g were found to prolong both PT and APTT significantly in a concentration dependent manner (Table 6.3). With increasing extent of steaming from 2 to 9 h, the PT and APTT for the steamed samples were increased significantly. Comparisons of raw and steamed samples showed that the PT and APTT for steamed samples were significantly longer than the raw samples at the various concentrations ($p<0.05$) tested. These results were further verified when a typical pair of *P. notoginseng* CPM, which have different chromatographic profiles (Chapter 3), were investigated. The steamed CPM was found to have prolonged PT (27 s) and APTT (81 s) as compared to the PT (21 s) and APTT (44 s) in the corresponding raw sample. On the other hand, for an atypical pair of CPM which gave similar chromatographic profiles, both PT and APTT were not significantly different between the two forms. In view of these results, the differences in the different types

of chemical components present in raw and steamed *P. notoginseng* have affected their anticoagulation effects. Differentiation between the two forms is hence important.

Table 6.3. Effects of extracts of raw and steamed *P. notoginseng* on PT and APTT in human blood plasma *in vitro* (n≥3)

Sample	Final conc. (mg/ml)	PT (s)	APTT (s)
Control	-	15.1 ± 0.7	42.2 ± 1.5
Raw *P. notoginseng*	3.3	17.7 ± 1.3*	44.4 ± 0.7
Steamed *P. notoginseng* (2h)	3.3	20.3 ± 1.3*	51.0 ± 3.6*
Steamed *P. notoginseng* (6h)	3.3	25.9 ± 0.9*	64.1 ± 4.0*
Steamed *P. notoginseng* (9h)	3.3	27.1 ± 0.8*	76.1 ± 4.0*
Raw *P. notoginseng*	5.0	20.6 ± 0.8*	43.7 ± 1.0
Steamed *P. notoginseng* (2h)	5.0	22.0 ± 1.5*	60.7 ± 5.0*
Steamed *P. notoginseng* (6h)	5.0	33.3 ± 1.8*	135.9 ± 1.9*
Steamed *P. notoginseng* (9h)	5.0	40.6 ± 1.8*	NC
Raw *P. notoginseng*	6.7	23.4 ± 1.0*	47.0 ± 1.3*
Steamed *P. notoginseng* (2h)	6.7	29.5 ± 0.5*	81.1 ± 4.2*
Steamed *P. notoginseng* (6h)	6.7	41.4 ± 1.3*	NC
Steamed *P. notoginseng* (9h)	6.7	58.1 ± 6.7*	NC

NC: no coagulation
*: $p<0.05$ compared to control

The APTT values for steamed samples were equivalent to various concentrations (0.017-0.167 U/ml) of heparin, which served as a positive control. Heparin, a clinically used anticoagulant drug, binds to antithrombin III (AT-III), which results in its enhanced affinity to several activated forms of factors, namely Factors IIa (thrombin), Xa, IXa, and this binding renders the factors inactive. Therefore, heparin was found to prolong APTT, but PT was only prolonged at much higher concentrations of heparin (Figure 6.5). This observation was also found in another study [Yun *et al*., 2001b]. The observation that both PT and APTT were prolonged by raw and steamed *P. notoginseng* suggested that the samples may act on a multitude of different factors in both the intrinsic and extrinsic pathways or they may affect the factors in the common coagulation pathway such as Factors II, V, X, fibrinogen.

A determination of thrombin time (TT) was further carried out for all the samples used in this study. Heparin showed an increase in TT with increasing concentrations. Furthermore, the extracts of *P. notoginseng* generally prolonged TT in a concentration dependent manner (Figure 6.6). Similar to the results of PT and APTT, the same trend whereby steamed samples gave longer TT than the raw samples, was observed. Earlier, it was shown that steamed samples prolonged PT and APTT more than raw samples. Likewise, steamed samples were found to prolong TT to a greater extent than raw samples. The pairs of CPMs also gave similar results.

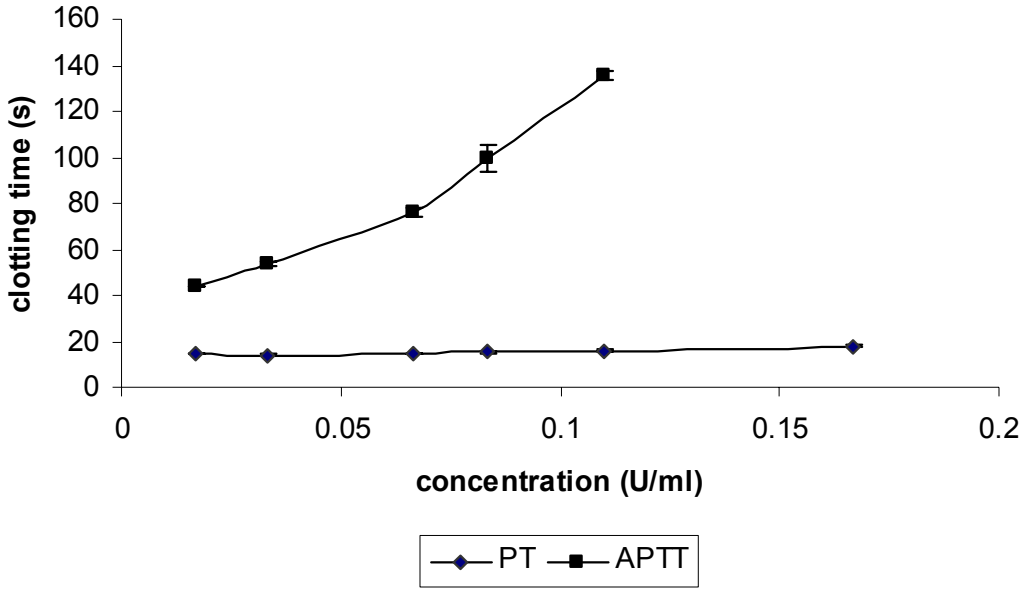


Figure 6.5. Effects of different concentrations of heparin on PT and APTT (n≥3).

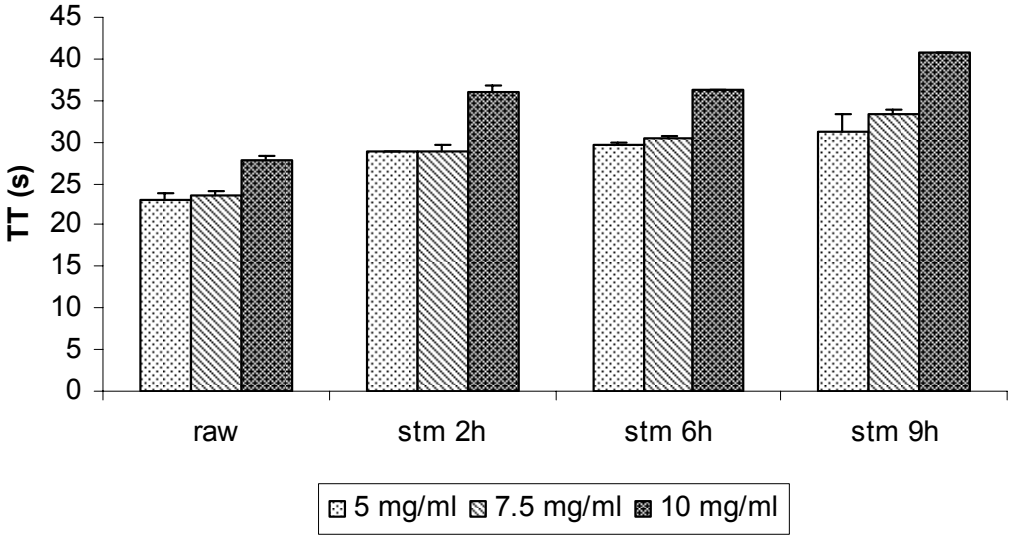


Figure 6.6. Graph showing the effects of raw and steamed *P. notoginseng* on thrombin time *in vitro* (n≥3).

In view of the above results, the effects of *P. notoginseng* may seem similar to high concentration of heparin, whereby all three of the above parameters were prolonged. Reptilase time can be used for the differential diagnosis of a prolonged TT. Presence of prolonged TT and normal reptilase time is usually used for the detection of exogeneous anticoagulant such as heparin. As supported by this study, the six different concentrations (0.017-0.167 U/ml) of heparin did not significantly change the reptilase time as compared to control (18.0 ± 0.5 s). Similar to heparin, the reptilase time was not significantly ($p>0.05$) prolonged using the same concentrations of *P. notoginseng* samples as in the TT assays. The results indicated that *P. notoginseng* samples did not affect the ability of fibrinogen to be cleaved by *Bothrops atrox* toxin, resulting in the formation of fibrin. Therefore, the samples may be directly or indirectly affecting the thrombin or have heparin-like effects. Presence of fibrinogen degradation products (FDP) from fibrinolysis will also increase TT and reptilase time. Since reptilase time was not prolonged, fibrinolysis may not have occurred. For further confirmation, the determination of fibrinogen in the plasma was carried out. A standard curve was developed and used for the determination of fibrinogen in *P. notoginseng*, its fractions, *P. ginseng* and *P. quinquefolium*. All the results showed that the concentration of fibrinogen did not change significantly ($p>0.05$) as compared to control (Table 6.4). Therefore, this confirmed that the samples did not cause degradation of fibrinogen or promote the fibrinolytic system.

Table 6.4. Effects of extracts of raw and steamed *P. notoginseng* on fibrinogen concentration in plasma (n≥3)

Sample	Fibrinogen (mg/dl)
Control	171.3 ± 6.2
Raw *P. notoginseng*	166.1 ± 4.3
Steamed *P. notoginseng* (2h)	159.9 ± 7.5
Steamed *P. notoginseng* (6h)	160.6 ± 9.6
Steamed *P. notoginseng* (9h)	175.1 ± 9.8
Butanol fraction	169.0 ± 2.8
Water fraction	165.4 ± 5.1
Hexane fraction	159.3 ± 6.7
Standard raw *P. notoginseng*	164.0 ± 8.4
P. ginseng	166.1 ± 2.6
P. quinquefolium	162.9 ± 8.5
Korean red ginseng	156.0 ± 8.5
Red ginseng	166.0 ± 4.2

***In vitro* blood coagulation assays of extracts of different *Panax* species**

For the other related *Panax* species, namely *P. ginseng* (Chinese), *P. quinquefolium* and red ginseng reference standard, PT was slightly prolonged compared to controls, but the PT were shorter than those obtained from raw *P. notoginseng* at the same concentration (Table 6.5). TT of these samples also showed some prolongation. Previous study has reported that red ginseng prolonged PT and

APTT [Yun *et al*., 2001b]. In addition, it was found to have an inhibitory effect against experimental disseminated intravascular coagulation in rats, and promoted fibrinolytic system in fibrin plates [Matsuda *et al*., 1986a; 1986b]. The active component was subsequently found to be ginsenoside Ro [Matsuda *et al*., 1986a; 1986b]. Ginsenoside Ro was not studied in this work as it has not been detected in *P. notoginseng*. In general, this study found that both raw and steamed *P. notoginseng* samples have more promising anticoagulation effects than the other *Panax* species.

Table 6.5. Effects of extracts of various *Panax* species on PT and APTT of human blood plasma *in vitro* (n≥3)

Sample	Final concentration (mg/ml)	PT (s)	APTT (s)
P. quinquefolium	5.0	17.3 ± 1.0	40.9 ± 0.4
P. ginseng	5.0	16.6 ± 1.1	41.4 ± 0.3
Korean red ginseng	5.0	14.6 ± 0.5	40.1 ± 0.8
Red ginseng reference standard	5.0	16.4 ± 0.9	40.3 ± 2.5
Raw *P. notoginseng*	5.0	20.6 ± 0.8*	43.7 ± 1.0
Steamed *P. notoginseng* (9 h)	5.0	40.6 ± 1.8*	NC

NC: no coagulation

In vitro* blood coagulation assays of fractions of *P. notoginseng

The fractions of steamed *P. notoginseng* were screened to determine the most active fraction for future identification of active compounds. Butanolic fraction prolonged both PT and APTT in a concentration dependent manner, as shown in Table 6.6. The water fraction did not prolong PT but prolonged APTT slightly (49.9 s) at a higher concentration (3.33 mg/ml). On the other hand, the hexane fraction did not prolong PT, but prolonged APTT significantly and to a greater extent than butanolic fraction. This may imply that the active components for anticoagulation activities are likely to be less polar components present in the hexane fraction and butanol fraction. These results were in agreement with a study on *P. ginseng* which found that the lipophilic fraction prolonged both the thrombin time and APTT in rats [Park *et al*., 1996].

Table 6.6. Effects of fractions from steamed *P. notoginseng* on PT and APTT of human blood plasma *in vitro* (n≥3)

Fractions	PT (s) at different concentrations			APTT (s) at different concentrations		
	0.8 mg/ml	1.7 mg/ml	3.3 mg/ml	0.8 mg/ml	1.7 mg/ml	3.3 mg/ml
Butanol	19.4 ± 0.2	35.7 ± 2.3	53.6 ± 2.7	45.9 ± 0.4	56.9 ± 0.3	NC
Water	14.5 ± 0.1	14.5 ± 0.3	16.4 ± 0.3	40.6 ± 0.4	46.2 ± 1.3	49.9 ± 1.1
Hexane	14.5 ± 0.3	15.1 ± 0.3	16.9 ± 0.8	69.6 ± 1.7	108.2 ± 1.7	NC

NC: no coagulation

***In vitro* blood coagulation assays of individual compounds**

Sixteen pure components present in raw and steamed *P. notoginseng* were evaluated for their effects on blood coagulation. The results are shown in Table 6.7. The shaded components are only present in the steamed samples. Ginsenosides Rk1 and Rg5 were found to prolong PT, while 20S-ginsenoside Rh1 prolonged APTT at a final concentration of 333 μM. In addition, these three ginsenosides were present only in the steamed samples. Individually, most of the other chemical components did not prolong PT and APTT, suggesting that these saponins were not the components responsible for the anticoagulation effects or the concentration was still insufficient. Another reason may be the lack of synergistic effects when the components were tested individually. Therefore, this work provided the first information on the anticoagulation activities of raw and steamed *P. notoginseng* extracts, fractions and pure components, so that future studies may further explore if there are other components responsible for these anticoagulation activities and their mechanisms.

Table 6.7. Effects of some chemical components in raw and steamed *P. notoginseng* on the PT and APTT in human blood plasma *in vitro* (n≥3)

Sample	Final conc. (μM)	PT (s)	APTT (s)
Control	-	15.1 ± 0.7	42.2 ± 1.5
Notoginsenoside R1	333	15.1 ± 0.1	42.5 ± 0.5
Ginsenoside Rg1	333	15.2 ± 0.2	42.2 ± 0.5
Ginsenoside Re	333	15.5 ± 0.2	42.7 ± 0.2
Ginsenoside Rb1	333	15.6 ± 0.6	40.7 ± 0.4
Ginsenoside Rc	333	16.7 ± 0.3*	43.7 ± 1.3
Ginsenoside Rd	333	14.5 ± 0.3	40.6 ± 1.4
20S-ginsenoside Rh1	333	15.7 ± 0.2	45.5 ± 1.1*
20R-ginsenoside Rh1	333	15.4 ± 0.2	43.2 ± 1.9
Ginsenoside Rk3	333	15.7 ± 0.1	43.1 ± 0.9
Ginsenoside Rh4	333	15.6 ± 0.2	43.2 ± 0.7
Ginsenoside Rk1	333	16.5 ± 0.4*	41.6 ± 1.0
Ginsenoside Rg5	333	16.1 ± 0.8*	42.8 ± 1.3
20S-ginsenoside Rg3	333	15.7 ± 0.6	42.5 ± 1.6
20R-ginsenoside Rg3	333	15.1 ± 0.2	42.8 ± 1.2
Dencichine	333	15.0 ± 0.4	42.5 ± 1.7
Diaminopropionic acid	333	14.8 ± 0.4	42.6 ± 1.1

Unshaded: Chemical components present in raw and steamed *P. notoginseng* with the steamed form having lower quantities. Shaded: Chemical components present in steamed *P. notoginseng* only.
*: $p<0.05$ compared to control

6.3 *In vivo* and *ex vivo* studies

6.3.1 Experimental

6.3.1.1 Materials

The raw *P. notoginseng* roots (sample 2) were obtained from Singapore (Section 3.1.2.1). Dencichine and aspirin were purchased (as mentioned in Section 5.2.1 and 6.2.1.1). All materials used for blood collection, platelet aggregation and blood coagulation experiments were also described in Section 6.2.1.1.

6.3.1.2 Animals

Male Sprague-Dawley rats (200-300 g) were purchased from Laboratory Animal Centre, Singapore. The care of the animals and the experiments carried out on the rats were in accordance to internationally accepted guidelines on laboratory animal use and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore. They were housed in an air-conditioned room (temperature of 22 ± 1°C and a humidity of 62 ± 2%) at the Animal Holding Unit of National University of Singapore. The rats were allowed unrestricted access to food (commercial rat pellet diet) and water and they were acclimatised for at least a week before any experimentation.

6.3.1.3 Sample preparation

Five g of each powdered sample were accurately weighed and extracted with 50 ml of 70% v/v methanol by ultrasonication for 20 min. The extracts were filtered and the ultrasonication process was repeated for two additional times. The combined filtrate was evaporated to dryness. The residue was weighed and suspended in appropriate volumes of 0.5% carboxymethylcellulose (CMC) solution. Steamed *P.*

notoginseng samples were steamed at 120°C for 9 h, using the method described in Section 3.1.2.3, before extraction by ultrasonication.

6.3.1.4 *In vivo* bleeding model

The samples were suspended in 0.5% CMC. The rats were divided into five groups: control (0.5% CMC), aspirin (25 mg/kg), dencichine (25 mg/kg), raw *P. notoginseng* (500 mg/kg) and steamed *P. notoginseng* (500 mg/kg). Each group consisted of at least 7 rats. The rats were fed with the appropriate sample solution by oral gavage. The concentration of samples was adjusted appropriately so that the total volume was not more than 3 ml for each rat. After 90 min, they were anaesthetised with a mixture of 75 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally. The tail bleeding model was modified from those of White *et al*. [2001] and Beviglia *et al*. [1993]. The tail was initially warmed for 3 min in a 0.9% saline solution which was previously warmed and maintained at 37°C. Bleeding was induced by transection of the rat tail at 5 mm from the tip. The distal portion of the tail (3 cm) was immersed vertically into the 0.9% saline solution at 37°C. The time between the start of transection to bleeding cessation was recorded as the bleeding time. Bleeding cessation was considered to be the time when blood flow stopped for at least 30 s. If the bleeding was not ceased by 15 min, the experiment was stopped and pressure was applied to stop the bleeding.

6.3.1.5 *Ex vivo* platelet aggregation assays

The rats were orally fed with the sample solutions and they were divided into five groups, each with at least 7 rats, similar to the above bleeding assays. After 110 min, 4 ml of blood was collected intra-cardiacally (by cardiac puncture) from the

anaesthetised rats into the citrated tube. A portion of the collected blood was used in platelet aggregation assays while the rest was reserved for blood coagulation assays. The platelet aggregation assays were performed as described in Section 6.2.1.5, except that no test sample solution was added to the blood samples. After equilibrating the blood sample with PBS for 2 min, the reaction was immediately induced with collagen.

6.3.1.6 *Ex vivo* blood coagulation assays

The rats were orally fed with the sample solutions and they were divided into four groups (control, dencichine, raw and steamed *P. notoginseng*), each with at least 7 rats. After 110 min, 4 ml of blood was collected intra-cardiacally (by cardiac puncture) from the anaesthetised rats into the citrated tubes. A portion of the blood was centrifuged at 3000 *g* for 10 min to obtain the platelet poor plasma (PPP). It was ensured that the plasma was minimally contaminated with lysed red blood cells by slowly drawing the blood into the syringe. The blood coagulation assays (PT and APTT) were performed as described in Section 6.2.1.6, except for the direct use of the rats' plasma instead of pooled human plasma and no test sample solution was further added into the rats' plasma.

6.3.1.7 Statistical analysis

The experimental results were expressed as mean ± SD and statistical analyses were performed using Student's *t* test (for comparisons between two groups) and one-way analysis of variance (ANOVA) (for comparisons between three or more groups) with Tukey-Kramer post hoc test. Data were considered statistically significant when p values were <0.05.

6.3.2 Results and Discussion

6.3.2.1 Bleeding time assays

A bleeding time (haemorrhagic) model was optimised and used in this study. Using this model, seven control rats were given CMC and the average tail bleeding time was 196.4 ± 44.7 s. This tail bleeding time of rats determines the time taken for the formation of a haemostatic plug in which platelets, plasma factors and blood vessel walls are all involved in the haemostasis process. It has often been used as a haemorrhagic model for determining primary haemostasis, detection of platelet disorders and the testing of antiplatelet drugs. The design of these models has been studied and many experimental factors will affect the bleeding times [Dejana *et al*., 1982]. These include the location, size, depth of the lesion, position of the tail (horizontal or vertical), environment, temperature and type of anaesthesia. Therefore, careful standardisation of the method is needed. Template bleeding models used specific small lesions on the dorsal part of the rat tail but it was unable to detect aspirin-induced platelet dysfunction [Dejana *et al*., 1982]. On the other hand, the bleeding model employing the transection of tail tip will involve the larger blood vessels in addition to the small ones in the dermis and subcutaneous tissues. Therefore, this bleeding time will be sensitive to both platelet function and coagulation defects and this latter model was more widely established than the former. There are numerous factors which can affect these pathways and some may have opposing or synergistic effects. Therefore, this bleeding time (haemorrhagic) model provides a measure for screening the overall platelet aggregation and blood coagulation effects *in vivo*.

Aspirin, being an antiplatelet drug, was used as a positive control for comparison with the sample-treated rats. Dosages of 10 to 200 mg/kg have been

employed in other *in vivo* studies [Matsuda *et al*., 1986b; Su *et al*., 1996; Dejana *et al*., 1982]. Preliminary studies with 10-50 mg/kg of aspirin in this rat bleeding model showed that aspirin positively prolonged the bleeding times in a dose-dependent manner. It prolonged the bleeding time to 605 s ($p<0.05$) at 25 mg/kg, which was significantly different from negative control. A dosage of 50 mg/kg prolonged the bleeding time to more than 15 min.

The bleeding time for rats given dencichine was slightly shorter but not significantly different from that obtained for control rats. This further supported the findings [Zhao and Wang, 1986] that dencichine was one of the haemostatic components that can reduce bleeding time in *P. notoginseng*. The ED_{50} for haemostasis was reported to be 35 mg/kg [Ishida *et al*., 1989] but all the previous haemostatic studies on dencichine were carried out using intraperitoneal administration. The different routes of administration and bleeding models may also result in some differences in bleeding times. The oral route was used in this study, as these herbs are usually orally consumed by humans. As found in Chapter 5, raw *P. notoginseng* contained more dencichine than the steamed form, so the raw form may have greater haemostatic effects.

As shown in Table 6.8, both raw and steamed *P. notoginseng* significantly prolonged the bleeding times to 397 s and 529 s respectively, as compared to the control. The bleeding times for the whole extract of steamed *P. notoginseng* and aspirin were not significantly different. Steamed *P. notoginseng* (oral administration of 500 mg/kg) effects on the prolongation of bleeding were comparable to that of aspirin (25 mg/kg), implying that it has good anti-haemostatic effects *in vivo* in a mammalian physiological system. At the same dosage, the bleeding times of raw and steamed *P. notoginseng* were further found to be significantly different from each

other. Steamed *P. notoginseng* has more potent anti-haemostatic effects than the raw form, as seen from the longer bleeding times. Due to the different potency or degree of haemostatic effects, it is therefore important to differentiate the raw and steamed forms. The saponins have antiplatelet effects as seen earlier from the *in vitro* studies and may play a role in prolonging the bleeding time *in vivo*. Dencichine, however, was found to have opposing effects and shorten bleeding times *in vivo*. Herbs may contain many components which may have different, opposing, cumulative or synergistic effects. The therapeutic effects of herbs would therefore depend on the concentration of the individual components and their complex effects on each other when mixed together in different proportions in the herb. For *P. notoginseng*, this study showed that the extracts of the herb administered orally resulted in significant anti-haemostatic effects *in vivo*. *Sophora japonica* is another example which was found [Ishida *et al*., 1989] to have an anti-haemostatic component (isorhamnetin) which can specifically suppress the haemostatic component (quercetin). The parched (heated on hot plate, 200°C, 2h) and unparched *Sophora japonica* also showed differences in haemostatic effects. Therefore, it is not uncommon to have both anti-haemostatic and haemostatic components present in the same herb and their complex mechanisms are largely not known.

There were two previous reports [White *et al*., 2000; White *et al*., 2001] on the effects of raw *P. notoginseng* on bleeding times using a haemorrhagic rat model. Contrary to our results, the alcoholic extracts of *P. notoginseng* were found to result in the shortest bleeding times, followed by the hydrophilic (water) extracts and lipophilic (hexane) extracts. Haemostatic effects were significant compared to placebo (wheat flour) for the alcoholic extract (which contains mainly saponins), but the other extracts were not significantly different from the placebo. The mechanisms of

haemostasis for *P. notoginseng* were not exactly clear. For both studies, the authors investigated its effects by applying the extracts externally or topically. This may have resulted in the different effects as compared to oral administration. The effects of oral administration of the steamed extract on bleeding times have not been previously reported. This may also explain why *P. notoginseng* has been traditionally used for cessation of bleeding, as well as for improving blood circulation and removing blood stasis - two seemingly opposing effects - but may be important in maintaining the overall balance and homeostasis of the blood system.

Table 6.8. Effects of oral administration of aspirin, dencichine, raw and steamed *P. notoginseng* on rats' tail bleeding time (*in vivo*) (n=7)

Treatment groups	Dosage (mg/kg)	Bleeding time (seconds)
Control (0.5% CMC)	-	196.4 ± 44.7
Aspirin	25	604.9 ± 84.0*
Dencichine	25	189.3 ± 43.9
Raw *P. notoginseng*	500	397.1 ± 38.2*
Steamed *P. notoginseng*	500	529.1 ± 78.7*

*: $p<0.05$ as compared to control rats, one-way ANOVA

6.3.2.2 *Ex vivo* platelet aggregation assays

The inhibitions of platelet aggregation after oral administration of samples into Sprague-Dawley rats are shown in Table 6.9. The results were consistent with the *in vitro* results. Dencichine resulted in low inhibition of platelet aggregation, while aspirin showed a 64% inhibition. Steamed *P. notoginseng* resulted in significantly higher % inhibition of platelet aggregation as compared to raw *P. notoginseng*. The percentage inhibition due to steamed sample at a dose of 500 mg/kg was also not significantly different from aspirin (25 mg/kg), implying that its effect was close to that of a commonly used antiplatelet drug. Therefore, steamed *P. notoginseng* may have potentially useful clinical effects which warrant further research. In this study, the dosages of aspirin administered were in the effective dose ranges that have been previously reported for platelet studies. The dosages of the extracts administered were also chosen based on the dose ranges that have been commonly used in other pharmacological studies. The above results showed that the inhibitors of platelet aggregations were able to reach the blood stream after oral administration and produced the effects similar to that obtained *in vitro*.

Based on the *in vitro* results, several of the individual saponins gave good activities, and their effects can be synergistic or cumulative when they were present together in the extracts. They were the likely components responsible for the *ex vivo* antiplatelet activities. This was further supported by studies which showed inhibition of platelet aggregations by *P. notoginseng* total saponins [Ma and Xiao, 1998] and *P. notoginseng* panaxatriol saponins [Su *et al*., 1996] given intraperitoneally to rats. A clinical study [Wang *et al*., 2004] in patients showed that raw *P. notoginseng* total saponins (Xuesaitong capsule) (120 mg twice daily for 4 weeks) reduced platelet activation, adhesion and aggregation, prevented thrombosis and improved

microcirculation. Its therapeutic effect on clinical syndrome was more effective than aspirin (50 mg once daily for 4 weeks). Mixtures of *P. notoginseng* and *Salvia miltiorrhiza* (danshen) of different ratios were also reported for its *ex vivo* platelet aggregation effects [Liu *et al*., 2002]. Among the various proportions of *Salvia miltiorrhiza* and *P. notoginseng* tested, it was found that a ratio of 10:3 was the best, while raw *P. notoginseng* extract alone gave less effect on rabbits' platelet aggregation. However, raw *P. notoginseng* alone could markedly inhibit platelet adhesion. Therefore, it is proposed that *P. notoginseng* may act through multiple components and multiple pathways.

Table 6.9. Effects of oral administration of aspirin, dencichine, raw and steamed *P. notoginseng* on platelet aggregations in rats *ex vivo* (n≥7)

Treatment groups	Dosage (mg/kg)	% inhibition
Aspirin	25	66.9 ± 19.9
Dencichine	25	8.0 ± 1.9
Raw *P. notoginseng*	500	36.8 ± 10.6
Steamed *P. notoginseng* (9h)	500	52.7 ± 15.2

6.3.2.3 *Ex vivo* blood coagulation assays

The PT and APTT in rats' plasma after oral administration of the samples are shown in Table 6.10. PT and APTT were not significantly changed in the rats that were given dencichine, raw and steamed *P. notoginseng*, as compared to control rats. The results did not agree with the *in vitro* results obtained in Section 6.2.2.2. There may be a few possible reasons for the observation. The bioavailability of the components responsible for anticoagulation activities may be low, so the concentration reaching the blood stream may not be sufficient enough to prolong the coagulation times *in vivo*. The dosage of the extracts administered to the rats, which contain a mixture of numerous components, may not be sufficiently high to overcome the low bioavailability. Alternatively, the active compounds may be metabolised by enzymes or rendered inactive in the physiological system. Pharmacokinetics of some saponins in *P. ginseng* such as ginsenosides Rg3, Rg1, Rb1, had been previously studied and they were found to have relatively low absorption and were metabolised upon oral administration [Xu *et al*., 2003; Tawab *et al*., 2003]. The pharmacokinetics of most components in *P. notoginseng* are still largely unknown. From this study, three ginsenosides (20S-Rh1, Rk1 and Rg5) were shown to have some anticoagulation effects *in vitro*. However, besides saponins, there may be other active components that may contribute to the anticoagulation effects. To add to the complexity, like most herbal medicines, the various components may work together synergistically to give its overall effects. *Ex vivo* coagulation studies on *P. notoginseng* have not been reported prior to this work. From the results of this study, for the same concentration of raw and steamed *P. notoginseng*, the *in vivo* bleeding times for both were significantly prolonged and antiplatelet effects were observed *ex vivo*, but *ex vivo* anticoagulant effects were not apparent. This demonstrated that the *in vivo*

prolongation of bleeding time in rats may be contributed by the antiplatelet effects more than the effects on the coagulation pathways.

Table 6.10. Effects of oral administration of dencichine, raw and steamed *P. notoginseng* on plasma coagulation parameters in rats *ex vivo* (n≥7)

Treatment groups	Dosage (mg/kg)	PT (s)	APTT (s)
Control (0.5% CMC)	-	8.4 ± 0.2	17.7 ± 1.9
Dencichine	25	8.5 ± 0.2	18.4 ± 0.2
Raw *P. notoginseng*	500	8.4 ± 0.3	18.6 ± 0.8
Steamed *P. notoginseng* (9h)	500	8.3 ± 0.5	19.6 ± 2.0

6.4 Conclusion

The raw and steamed *P. notoginseng* roots were found to have antiplatelet (*in vitro* and *ex vivo*) and anticoagulation (*in vitro*) activities, with the steamed samples having significantly higher activities than the raw samples. These activities were also seen in other *Panax* species such as *P. ginseng* and *P. quinquefolium*, but to a lesser extent. Most of the saponins were found to have varying degrees of antiplatelet activities. The antiplatelet activities of steamed *P. notoginseng* were likely due to the cumulative effects of the saponins present. The IC_{50} values of ginsenoside Rg5 and 20S-ginsenoside Rg3 for the inhibition of collagen-induced platelet aggregation showed that these two ginsenosides were more potent than aspirin. They may serve as potential leads for antiplatelet drugs and further research is warranted.

In vivo bleeding times of rats orally-fed with the extracts were prolonged significantly and these were likely to be due to their antiplatelet effects. This study showed that *P. notoginseng* is a useful medicinal herb with antithrombotic activities, especially the steamed form which may have potential for further development of antithrombotic drugs. In addition, these results highlighted the impact of steam processing on *P. notoginseng* and the importance of standardising this process for consistent antithrombotic activities.

CHAPTER 7

CONCLUSIONS AND FUTURE PROSPECTS

Chapter 1 gives an overview of the importance, safety, efficacy and quality of herbal medicines, as well as the importance of antithrombotic and haemostatic therapies in cardiovascular diseases. Furthermore, many prescription drugs have botanical origin and plants are good sources of lead compounds. The root of *P. notoginseng* is an important and highly regarded Chinese medicinal herb, available in both raw and steamed forms. Their traditional uses differ and the ability to differentiate them is important. Prior to this work, there is a general lack of studies on the chemical and biological differences between the two forms.

Chapter 2 describes the rationale, hypotheses and objectives of this work. The main objectives are to develop methods for the quality control of *P. notoginseng*, and to study the effects of processing, namely, the chemical and biological differences between raw and steamed *P. notoginseng*.

Several new methods for the quality control of *P. notoginseng* are developed and discussed in Chapter 3. A HPLC-DAD analytical method (Section 3.1) was successfully developed for the simultaneous analysis of six saponins, namely, ginsenosides Rb1, Rc, Rd, Re, Rg1 and notoginsenoside R1. This method was applicable to the differentiation of the two different forms of *P. notoginseng*, as well as differentiating it from two other *Panax* species, namely, *P. ginseng* and *P. quinquefolium*. The entire extraction and analytical method has been optimised and validated for linearity, precision, specificity, LOD and LOQ. Coupled with hierarchical clustering analysis, the different species were distinctly clustered into different groups. Raw *P. notoginseng* samples from different sources were found to

have similar profiles but their saponins concentrations may vary considerably. It was also discovered that the individual roots from a single GAP source had several outliers with slightly different profiles and varying ratios of notoginsenoside R1, ginsenosides Rb1 and Rd. From the chemical studies, it was found that the concentrations of the analysed saponins were decreased upon steaming and additional components were formed. The differences increased significantly with increasing duration of steaming. Prior to the work, raw and steamed *P. notoginseng* have not been compared or analysed quantitatively using advanced analytical techniques. The chemical studies were extended to eleven pairs of raw and steamed *P. notoginseng* CPMs and discrepancies were found in three pairs, showing the current lack of quality control and standardisation of the process. The method developed will be useful for routine quality control of *P. notoginseng* samples.

In addition to assaying the saponins, another quality control measure is chromatographic fingerprinting. In Section 3.2, a novel HPLC method coupled with chromatographic pattern matching analysis was first developed to quantitatively compare the chromatograms of raw and steamed *P. notoginseng* samples. The average pattern match standard deviation values for identical samples were found to be below 0.0006. The pattern match standard deviation values increased with the duration of steaming and were statistically higher than that obtained for identical samples. These values gave useful quantitative indications of the similarity of the sample pairs and served as a similarity or match index. From these, the degree in which the raw root samples were changed by the steaming process was successfully deduced. This method was capable of detecting inconsistencies in the CPMs. This is the first application of the pattern matching method for differentiating herbs or complex samples based on their chromatographic profiles. The degree of similarity between

samples can be determined quantitatively and it is useful for standardising herbal processing. The HPLC method coupled with chromatographic pattern matching analysis is a potentially useful tool in ensuring quality of complex herbal samples.

In Chapter 4, major potential markers that play key roles in the differentiation of raw and steamed *P. notoginseng* were successfully isolated and purified. Their identities were confirmed using NMR and MS. Their purities were determined by HPLC and found to be >95% for all compounds. 20S-ginsenoside Rh1, 20R-ginsenoside Rh1, 20S-ginsenoside Rg3, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5 were identified. They were found to be predominantly present in the steamed form and their concentrations increased with increasing extent of steaming. To date, this is the first study of the detection and isolation of ginsenosides Rk1 and Rk3 from *P. notoginseng* roots, as well as the first separation and identification of these fourteen saponins in a single HPLC chromatogram of steamed *P. notoginseng*. Several constituents (20S-ginsenoside Rh1, 20R-ginsenoside Rh1, 20S-ginsenoside Rg3, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5) were not detected in the raw samples. From the determination of their structures and the corresponding reduction in the concentrations of some saponins in raw *P. notoginseng* upon steaming, it was apparent that the new components were formed from some saponins in the raw form. The new components were postulated to be formed by hydrolysis and loss of the glycosyl moieties, isomerisation and dehydration at C-20. Of the eight marker components, ginsenosides Rh1, Rg3, Rg5 and Rh4, have been individually reported in other studies to have anticancer effects, therefore implying that the steamed form may have different activities compared to its raw form. However, to date, the biological activities of ginsenosides Rk1 and Rk3 have not been reported. These constituents warrant further research as they are present in significant quantities

in steamed samples. Future studies should explore the minor saponins, as well as the other classes of compounds present in steamed *P. notoginseng*. The determination of the isolated compounds can also be done in the future to standardise their amounts in the steamed samples. In view of the differences in components and activities, it is evident from the results that the steaming process has an important impact on *P. notoginseng* and standardisation of the process is critical in ensuring consistent quality and efficacy of steamed products.

Besides saponins in *P. notoginseng*, dencichine is another important constituent, reported to be responsible for raw *P. notoginseng*'s main haemostatic indication. In Chapter 5, a novel HILIC/ESI-MS/MS method was developed for the analysis of underivatised dencichine in *Panax* species. This is the first report of analysis of dencichine using LC-MS/MS. Being a small polar amino acid derivative, its retention on various reversed-phase columns is difficult without derivatisation and its analysis by LC-MS is challenging. This novel use of HILIC separation method was found to be suitable for retaining underivatised dencichine and for coupling with MS. The developed method was rapid (5 min), selective, sensitive and did not require any sample derivatisation. Elution time was about 1 min. The method was validated for linearity, accuracy, intra- and interday precision, LOD and LOQ, and applied to the analyses of raw and steamed *P. notoginseng* root samples and such products. The method is useful for the determination of dencichine in the quality control of *Panax* species, and it will also be applicable to other complex food crops that may contain dencichine.

The dencichine concentrations in various raw *P. notoginseng* roots were found to range from 0.134 to 0.293% w/w. When taken in normal recommended dose, the amount of dencichine is safely below the neurotoxic dose. Results also showed that

when the duration of steaming increased from 2 to 9 h, the concentration of dencichine in *P. notoginseng* decreased significantly to a negligible amount. The change was monitored and determined to be due to the hydrolysis of dencichine to diaminopropionic acid.

Eleven pairs of raw and steamed *P. notoginseng* CPM products were also analysed for their dencichine concentration. Consistent with the above results on steamed *P. notoginseng* roots, most of the CPM steamed products had lower concentrations of dencichine than their corresponding raw products, but their differences varied according to the different extents of steaming. This further emphasised the importance of standardising the steaming process to ensure consistent concentration of dencichine. A higher dencichine concentration in raw *P. notoginseng* root indicated that it may have better haemostatic activity than the steamed samples. *P. ginseng* (0.021-0.033% w/w) and *P. quinquefolium* (0.018-0.024% w/w) were also found to have significantly lower dencichine concentrations than raw *P. notoginseng*. This may provide an explanation as to why the related *Panax* species and steamed *P. notoginseng* are not traditionally indicated for haemostasis. Therefore, this work provided a scientific basis for the haemostatic use of raw *P. notoginseng*. However, the detailed mechanism of action of dencichine's haemostatic activity has to be further elucidated in the future. The results from all the chemical studies support the hypothesis that processing of raw *P. notoginseng* by steaming changes the concentration and composition of chemical constituents in *P. notoginseng*. The chemical profiles of both forms can be differentiated from each other and from its closely related species, using various qualitative and quantitative methods.

Chapter 6 describes the biological studies on platelet aggregation and blood coagulation *in vitro* and *ex vivo*, in order to explore if the differences in chemical

components result in different biological activities. The raw and steamed *P. notoginseng* roots were found to inhibit collagen-induced platelet aggregations in a dose dependent manner. The steamed samples were significantly more potent than the raw samples and the percentage inhibition increased with the extent of steam processing. Related *Panax* species such as *P. ginseng* and *P. quinquefolium* were found to have lower activities. Red ginseng (i.e. processed *P. ginseng*) also demonstrated slightly higher inhibitory activities as compared to *P. ginseng*. The butanolic fractions of steamed *P. notoginseng* resulted in the greatest inhibition of platelet aggregation as compared to the hexane and water fractions, implying that the butanolic fraction contained most of the active constituents.

The individual saponins were studied and found to exhibit varying degrees of inhibition of platelet aggregation. The IC_{50} values for the two most active ginsenosides, namely, ginsenoside Rg5 and 20S-ginsenoside Rg3, were 98 μM and 92 μM respectively. They were found to be more potent than aspirin (IC_{50} 131 μM), thus they may serve as potential leads for the development of antiplatelet drugs. As most of the saponins studied in this work showed some platelet inhibition activities, the overall antiplatelet activities of steamed *P. notoginseng* may be due to the cumulative effects of all the saponins present. The detailed mechanisms for platelet aggregations of these active compounds and extracts have to be further explored in order to understand their mechanism of action.

Further *ex vivo* platelet aggregation studies were carried out, confirming the platelet inhibitory activities of both raw and steamed *P. notoginseng*. This result is consistent with that found in the *in vitro* studies. The inhibitory effects of the steamed samples were also demonstrated to be comparable to that of aspirin. This is the first study for the comparison of inhibition of platelet aggregation activities in raw and

steamed *P. notoginseng*, together with related *Panax* species. Several individual saponins, such as notoginsenoside R1, 20RS-ginsenoside Rh1, 20R-ginsenoside Rg3, ginsenosides Rk3, Rh4, Rk1 and Rg5, were also studied individually for the first time.

For coagulation studies, it was found that the extracts of raw and steamed *P. notoginseng* prolonged PT and APTT in a dose dependent manner and the coagulation times were significantly increased with the extent of steaming. For *P. ginseng*, *P. quinquefolium* and red ginseng (steamed *P. ginseng*), the resulting coagulation times were significantly shorter than that from raw *P. notoginseng* at the same concentration, showing that *P. notoginseng* has a better anticoagulation effect than the other *Panax* species. From the results obtained from the different fractions of *P. notoginseng*, it was found that the active components with anticoagulation activities were likely to be the less polar components present in butanol and hexane fractions. The individual saponins were tested and most were found to show weak or no activities. These results indicated that the saponins may not be the main components causing the anticoagulant effects. Other components present in *P. notoginseng* inhibiting blood coagulation should be further explored.

Preliminary assessment on the potential mechanisms of anticoagulation action was carried out by determining the thrombin time, reptilase time and fibrinogen, using various *P. notoginseng* extracts. The samples appeared to affect both the intrinsic and extrinsic coagulation pathways. However, they neither caused degradation of fibrinogen nor promoted the fibrinolytic system, but may have heparin-like effects. The detailed mechanisms of the extracts could be further explored. Raw and steamed *P. notoginseng* did not prolong the coagulation times *ex vivo*, nevertheless, this is the first study on their coagulation effects and future studies need to further investigate their pharmacokinetics after oral administration.

An *in vivo* haemorrhagic rat model was used to determine the overall haemostatic ability. This model provided a measure for screening the overall *in vivo* platelet aggregation and blood coagulation effects. Dencichine was found to shorten bleeding time, hence demonstrating *in vivo* haemostatic activity. Bleeding times were prolonged significantly by orally administered raw and steamed *P. notoginseng* extracts when compared to negative controls. Results demonstrated that the steamed form was significantly a stronger anti-haemostatic than the raw form, and its effect was comparable to that of aspirin at a dosage of 25 mg/kg. This was consistent with the results that the raw form contained a higher concentration of dencichine, a haemostatic agent. These results indicate that there are many components present in *P. notoginseng* which may have complex and opposing effects. However, from this study, the overall effects after oral administration of raw and steamed *P. notoginseng* extracts were found to be anti-haemostatic *in vivo*.

The biological studies carried out supported the hypothesis that the changes in chemical constituents upon steam processing have significant effects on the biological activities, such as platelet aggregation, blood coagulation and haemostasis. These studies showed that *P. notoginseng*, especially the steamed form, would be a useful medicinal herb with promising overall antithrombotic activities that may prevent thrombosis in patients. The two potent saponins (ginsenoside Rg5 and 20S-ginsenoside Rg3) may also be promising leads which warrant further detailed mechanistic studies. In view of these results, the presence of any important herb-drug interactions with clinically used antithrombotic agents would need to be evaluated. Healthcare professionals should be cautious about these potential interactions which may occur in patients consuming *P. notoginseng* together with other antithrombotic

drugs. Besides antithrombotic activities, other activities such as anticarcinogenic or chemopreventive activities may also be worth exploring in the future.

In conclusion, the quality control of *P. notoginseng* to ensure proper differentiation between its raw and steamed forms is important, as steam processing has important implications on its activities. The work in this thesis is one step towards understanding botanical medicine. The chemical and biological differences between the two different forms of *P. notoginseng* and related species have been scientifically evaluated. Further scientific studies can be carried out to further understand their traditional uses, explore other potential new uses for this traditional herb, elucidate the mechanism of action and to discover potential new drugs.

The methods developed in this work can also be extended to the quality control of other botanical medicine, to enhance the quality, safety and efficacy of such natural remedies. With further understanding of the effects of processing on the chemical components and biological activities, the full benefits of botanical medicine can be potentially reaped by ensuring high quality and safety, to meet unmet therapeutic needs.

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