Infrared-based technique for the analysis of pharmaceutical Ginseng products

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Infrared-based Technique for the Analysis of Pharmaceutical Ginseng Products

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ABSTRACT

Quality of pharmaceutical products like ginseng is important for ensuring consumer safety and efficacy. Many ginseng products sold today are in various formulations which render it less identifiable by smell, taste or physical appearance. Furthermore, as ginseng is expensive, adulteration with other cheaper products occurs. Hence quality assurance of ginseng is needed. Since different chemical constituents in herbs like ginseng tend to exhibit characteristic infrared (IR) fingerprints, the aims of this study are thus to assess the feasibility and establish an analytical strategy for the use of IR spectral wavelengths in distinguishing different species of ginseng, as well as from adulterants, via mathematical processing of their spectra into derivatives and chemometric analysis using principal component analysis (PCA) modeling techniques. This has not been done before by others at the time of writing this report.

The samples used in this study included ginsenoside standards, American (*Panax quinquefolius*) and Asian (*Panax ginseng*) ginsengs, *Platycodon grandiflorum* (jiegeng), sawdust, as well as three commercial ginseng products – American ginseng capsules, Korean ginseng capsules, and American ginseng tea.

Ginsenoside standards, American and Asian ginseng root samples were obtained locally and analyzed using a Fourier transformed infrared (FTIR) spectrometer (Shimadzu Corporation Pte. Ltd., Asia Pacific) measuring in the mid-infrared ($4000\text{--}400\text{cm}^{-1}$) and near-infrared ($7800\text{--}4000\text{cm}^{-1}$) ranges. Their IR spectra were compared with each other, as well as with adulterants and commercial products based on spectral similarity and chemometrics via PCA modeling of the spectral data.

Based on the results of the present study, the two species of ginseng (*Panax quinquefolius* and *Panax ginseng*) could be differentiated from each other based on the techniques used in this work for both qualitative and quantitative analysis of their spectral fingerprints. The American and Asian ginsengs could be clearly distinguished from sawdust and *Platycodon grandiflorum*, as shown from the different groups in their two-dimensional score plots. PCA modeling of the IR spectra could also differentiate among the various grades of ginseng in Singapore. Further establishment of the infrared-based technique was also demonstrated through the analysis of three grades of ginseng samples from Hong Kong and the three commercial ginseng products.

In conclusion, the results of the present study show that the use of infrared wavelengths, together with the deterministic PCA method introduced here for the analysis of ginseng products has proven to be appealing. A novel quality sensing strategy based on mathematical processing of infrared wavelengths, current or enhanced mathematical algorithms, as well as other physical or pharmacological parameters, could potentially be developed for the detection of pharmaceutical products such as ginseng.

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LIST OF ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
ATR	Attenuated Total Reflection
BAC	Bacterial Artificial Chromosome
BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
CID	Collision-Induced Dissociation
DAD	Diode Array Detection
DAMD	Direct Amplification of Minisatellite region DNA
DNA	Deoxyribonucleic Acid
IC-PAD	Ion Chromatography separation with Pulsed Amperometric Detection
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detector
ESI-MS	Electrospray Ionization Mass Spectrometry
ESTs	Expressed Sequence Tags
FIR	Far-infrared
FPLC	Fast Protein Liquid Chromatography
Frit-FAB	Frit-fast atom bombardment
FTIR	Fourier Transformed Infrared
GC	Gas Chromatography
HPLC	High-Performance Liquid Chromatography
MEKC	Micellar Electrokinetic Chromatography

MIR	Mid-infrared
MLAPS	Multi-light Addressable Potentiometric Sensor
MS	Mass Spectrometry
NIR	Near-infrared
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Regions
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SPR	Surface Plasmon Resonance
TCM	Traditional Chinese Medicine
TLC	Thin-Layer Chromatography
TSP	Thermospray
US FDA	United States Food and Drug Administration
UV	Ultraviolet
2-DE	Two-dimensional Gel Electrophoresis

CHAPTER 1

INTRODUCTION

Since historical times, the Chinese have always believed that foods and medicines which are derived from plant and animal sources are good for health, and that a person's physical well-being is based on two principles: Yin and Yang [1]. This forms part of the concept of holism in Traditional Chinese Medicine (TCM) [2].

Ginseng is a famous Chinese herb [3-6] and two major varieties exist: *Panax ginseng* C.A. Meyer (Asian) and *Panax quinquefolius* L. (American) [5-12]. Asian ginseng is said to have a “warm” or “yang” property, while American ginseng has a “cool” or “yin” characteristic since the former has a more stimulating effect on the central nervous system [11-15]. Different parts and species of ginseng are believed to have different medicinal properties [12]. The ginseng root has been said to relief medical conditions involving the cardiovascular, endocrine, immune and central nervous systems [4,6,16-18]. It has also been used in the alleviation of diabetes, insomnia, fatigue, stress and shock, among other treatments [1,4,7,17,19-25]. Ginseng also exhibits an “adaptogenic” effect [17,26,27], and improves physical and mental performance [7,22,26]. A detailed list of ginseng effects is shown below:

- a) Alleviates fatigue, insomnia and stress.
- b) Antihyperglycemic, antioxidant and non-organ specific anticancer activities.
- c) Boosts general vitality.
- d) Improves learning and memory.
- e) Modulates cardiovascular activities and immune functions.
- f) Promotes hematopoiesis.

Herbal medicine is generally known to be effective and safe, and many people have benefited from such treatment [28]. However, there have been case reports [28,29] of adverse reactions with the use of certain herbs, like renal and liver failure, or even death. Many factors could contribute to these adverse reactions. Examples include drug interactions, hypersensitivity reactions, poor quality control, overdose and toxicity, as well as inconsistent prescribing practices [28]. It has been reported [30] that the ginseng abuse syndrome, characterized by depersonalization and confusion, could occur in individuals who had excessive intakes of ginseng. Other case reports of side effects [29] include deterioration in the conditions of schizophrenic patients, mania in a woman with a history of depression, reversible Stevens-Johnson syndrome, and cerebral arteritis. Thus, there must be a balance between safety and efficacy in the therapy of patients.

1.1. Objectives

In consideration of all the factors above, a novel analytical strategy based on western analytical techniques was defined for ginseng in the present study. Since infrared spectroscopic techniques offer the advantages of being fast, requiring no or little sample preparation, and producing no hazardous waste; and furthermore, the infrared spectra can be used as “fingerprints” for compound identification, the following objectives were laid out in the present research:

- a) To investigate the feasibility of infrared wavelengths as an analytical technique for ginseng authentication;
- b) To establish an analytical technique based on qualitative analysis and principal component analysis of infrared spectral fingerprints to characterize and

differentiate various species of ginseng, as well as from morphologically and chemically similar adulterants; and

- c) To further extrapolate the infrared-based technique to the authentication of commercial ginseng products.

1.2. Scope

The present study was initiated with the intention of providing the TCM and pharmaceutical manufacturing industries with a rapid novel sensing strategy for analysis of herbal medicines in order to complement the current “gold standard” chromatographic and mass spectrometric techniques. Ginseng was used in this work as an example. The potential of infrared spectroscopy in the analysis of TCMs is beginning to gain recognition in the pharmaceutical arena. Thus, this technique was selected.

This study focuses on the comparison between ginseng species and their products, as well as distinguishing them from two common adulterants (sawdust and *Platycodon grandiflorum*). This was in accordance with the western classification of ginsengs into the *Panax* genus. The rationale of focusing the present study based on “western methods” is because complementary and alternative medicine, which includes TCM, is currently not recognized yet as the dominant main stream system in the healthcare and medical world [31]. Even though certain TCM practices are gaining popularity in the West, many people still consider traditional medicine as “unproven” due to limited scientific evidence and lack of controlled clinical trials [31].

The two major ginseng varieties (Asian and American ginsengs) used for comparison in this study were purchased from Singapore and Hong Kong. Mr. Gary Pang, district manager of a major Chinese medicinal hall chain (Hockhua Ginseng Birdnest

Trading Enterprises, 福华人参燕窝贸易企业), confirmed that they grew their Asian and American ginsengs in specific plots of land which were exclusive to them in China, and that these ginsengs were not exported overseas but were for their own branches in Singapore [32]. This piece of news was especially relevant in this study since the current strategy of ginseng authentication would not be valid if the ginsengs from Hong Kong were from the same company.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1. The Value of Ginseng Quality

Ginseng is known as the king or lord of herbs [33]. Its genus name *Panax* is the Latin name for ‘panacea’ which means to ‘cure all illnesses’ in Greek (Pan = all, axos = medicine) [33,34]. The name ‘ginseng’ or ‘Ren shen’ (人參) is because its root is shaped like a human [33,34]. Another name for Asian ginseng is ‘Di Jing’ (地精) meaning the ‘essence of the earth’ because the purest part of the earth is believed to accumulate in the ginseng root, thus wild ginsengs are actually more valuable than cultivated ones [34]. American ginseng (西洋参) is also frequently nicknamed as ‘green gold’ because of its precious value. As with Asian ginseng, the wild variety is also the best [35]. It is also a traditional belief that as a ginseng grows in age, its efficacy also improves, hence it is not uncommon to hear of people who prefer to consume a three to five hundred-year-old Chinese ginseng root [35].

2.1.1. Traditional Methods of Authenticating the Quality of Ginseng

According to the quality standards set by the People’s Republic of China, there are three grades of ginseng based on factors such as moisture content and levels of ginsenosides [36]. The level of ginsenosides may vary according to species, growing environment and time of harvest [37]. Cultivation of ginseng is slow and difficult, taking more than 4 years before its roots can be harvested [38-40]. In Asia, ginseng quality is based on the origin of growth (e.g. in the wild or under cultivated conditions), age and physical characteristics of the root, rather than the level of ginsenosides [13,41]. The

major characteristics which define a good quality ginseng root are shown in Figure 2.1. The potency of ginseng roots is determined by its shape, which can be classified into three kinds based on the numbers and sizes of the lateral branches on the main root [36]: “pencil” roots have a slender body and do not have laterals; “chunky” roots have a thick, round body with a few large laterals; and “complex” roots do not have a central body, but consist of several branches, thus giving a “chicken claw” or “spider” shape. Large, chunky roots are usually preferred, and are of higher economic value than pencil roots [42]. In Singapore, the grading system is similar [41] with the ginseng roots being classified into ‘round-forked roots’, ‘long-straight roots’ and ‘small grain roots’ in decreasing order of grades.

Ginseng can be further classified based on their area of cultivation [41] into ‘wild’, ‘woods grown’, ‘woods transplanted’, ‘farm transplanted’ and ‘cultivated’ ginsengs, with the wild ginsengs being of the highest grade. It is commonly accepted that a higher grade ginseng root is usually more expensive. Thus, it can be inferred that a higher-priced ginseng should be of a better quality than a lower-priced one.

Figure 2.1. The major characteristics of a good quality Chinese ginseng root. Adapted from reference [34].

2.1.2. Role of Ginsenosides in Quality Assessment of Ginseng

The chemical composition of ginseng includes the dammarane saponins, naphtha class and carbohydrates [43]. American ginseng also contains a significant amount of starch [43]. The pharmacological activity of ginseng is not due to the presence of a single compound, but rather the mixture of its constituents [15,37]. It is accepted that the triterpene glycosides (saponins), called ginsenosides, are the major active ingredients in ginseng [3,5,8,9,22,23,25,26,44-46]. There are more than 30 known different ginsenosides [3,47-49], and they are characterized by a four trans-ring rigid steroid aglycone skeleton and attached sugar moieties [16,25,30]. The steroid component can be grouped into the 20-(S)-protopanaxadiols (e.g. Ra₁₋₃, Rb₁₋₃, Rc, Rd, Rg₃, Rh₂) and 20-(S)-protopanaxatriols (e.g. Re, Rf, Rg₁, Rg₂, Rh₁) [3,4,17,22,27,30,49-51], except for ginsenoside Ro [3,19] which is derived from oleanolic acid [4,17,19,22,27,48,50].

Figure 2.2 shows the major ginsenosides Rb₁, Rb₂, Rc, Rd, Re and Rg₁ which are used as markers of ginseng quality [8,9,30] because they account for 90% of the total ginsenosides [15,48,52]. The four malonyl derivatives of ginsenosides Rb₁, Rb₂, Rc and Rd, together with ginsenoside Ro, are termed the “acidic” ginsenosides, while the others are termed “neutral” ginsenosides [17]. The profiles of American and Asian ginseng can be distinguished by the gluco-ginsenoside Rf, which is detectable in Asian ginseng but not in American ginseng [9,17,19,23,26,30,36,50,52]; and the ocotillol type triterpene 24-(R)-pseudoginsenoside F₁₁ which is present in American ginseng but absent in Asian ginseng [30,50,52].

The relative abundances of ginsenosides can be used to distinguish between the Asian and American species [17,25]. A higher amount of the protopanaxadiol group of

ginsenosides exists in American ginseng, in contrast to a higher amount of the protopanaxatriol group in Asian ginseng [17,25]. Ginsenoside ratios are also indicative of the types of ginseng, even though they may differ among species. A higher Rb_1/Rg_1 value is usually indicative of *P. quinquefolius*, while a lower value indicates *P. ginseng* [15,17]. Furthermore, a high Rf/F_{11} ratio of more than 700:1 distinguishes Asian from the American species [17,52].

Figure 2.2. Structures of selected ginsenosides. Glc, glucose; Ara(p), arabinose (in pyranose form); Ara(f), arabinose (in furanose form); Rha, rhamnose; Ma, malonyl; GlcUA, glucuronic acid. Adapted with modification from references [17,25].

2.1.3. Importance of Quality in Herbal Supplements such as Ginseng

Quality of food, drug and pharmaceutical products is important for ensuring safety and efficacy to consumers and the U.S. Food and Drug Administration (FDA) ensures that these standards of safety, efficacy and quality are kept in these products [1,7]. However, most herbal products are not regulated because they are not considered as medicines but dietary supplements, unless the product is known to be harmful [15,53]. Herbs like ginseng have traditionally been authenticated by morphological and histological means, but these approaches are hardly reliable [11,54-56] because they are not only morphologically similar [11,52,54,56,57], but many commercial ginseng products today are also in various formulations such as powder, capsules, tablets, softgels, liquid extracts and tea [6,11,15,17,52,54,56], which makes it harder to identify by appearance, smell and taste [26]. Although there is a large range in ginsenosides concentration among the various types of ginseng products, the average concentration in descending order is in root powders, tea bags, dry roots, pastes, capsules, tablets, instant tea and liquid products [8]. Furthermore, as ginseng is an expensive herb, adulteration with other cheaper products occurs [23,26,28,44,52]. Adulterators are also continuously developing unique ways to make their desired product's chemical profile similar to that of the natural product [58]. Thus, various major techniques are required throughout the years to authenticate ginseng based on target marker molecules such as the level of ginsenosides.

2.2. Analytical Techniques

Several qualitative and quantitative analytical techniques have been developed by others for the evaluation of ginseng quality. These include high-performance liquid

chromatography (HPLC), mass spectrometry (MS), micellar electrokinetic chromatography (MEKC), column chromatography, thin-layer chromatography (TLC) and gas chromatography (GC), colorimetry, enzyme immunoassays, genomic and proteomic methods, as well as microchemical and spectrophotometric methods.

This section discusses the major techniques that have been used in the analysis of ginseng quality, as well as their potential advantages and limitations, to demonstrate the variety and evolution of methods that have been used in the authentication of ginseng throughout the years. Table 2.1 features a summarized list of common techniques used for the analysis of ginseng.

Table 2.1. Common techniques used for the analysis of ginseng.

Techniques	Features	Advantages	Disadvantages	Refs.
High Performance Liquid Chromatography (HPLC)	Can be combined with various interfaces such as ultraviolet (UV), evaporative light scattering detector (ELSD), fluorometry, diode array detection (DAD), thermospray (TSP) and frit-fast atom bombardment (Frit-FAB)	<ul style="list-style-type: none"> • Sensitive. • Able to analyze non-volatile, polar compounds. • ELSD is more sensitive than UV interface, fast, relatively cheap and straightforward. • Fluorescence detection is reliable and sensitive. 	<ul style="list-style-type: none"> • UV detection is limited to short wavelengths (201-205nm), and is less sensitive due to interference of other compounds with analysis. • TSP and Frit-FAB interfaces have poor reproducibility and lack the ability to detect malonyl-ginsenosides. • Indirect methods of detecting malonyl-ginsenosides have to be employed as there are no standards. • Methanol, a toxic chemical, is used for extraction of ginsenosides, hence is not acceptable for food processing. 	[3,8,17,27,44,45,59]
Mass Spectrometry (MS)	Can be used in combination with chromatography techniques. Methods of ionization/desorption include electrospray ionization (ESI), field desorption, secondary ion and electron impact ionization (EI). Collision-induced	<ul style="list-style-type: none"> • MS detector is more sensitive and specific when compared to UV or ELSD detection. • Results are not ambiguous, as MS can be used for confirmatory identification of ginsenoside peaks. • ESI method avoids tedious derivatization 	<ul style="list-style-type: none"> • Time consuming due to the need for chemical derivatization before analysis. • Too costly for use in routine analyses. 	[3,17,20,25,27,44,45,47,50,60]

	dissociation (CID) is used for ion fragmentation in tandem MS.	and separation steps, is sensitive, has better reproducibility, and can overcome problems associated with the malonyl-ginsenosides and low molecular ion abundance levels. <ul style="list-style-type: none"> • Structural information can be obtained when combined with CID method. 		
Gas Chromatography (GC)	Can be combined with MS technique.	<ul style="list-style-type: none"> • Sensitive and gives good resolution. 	<ul style="list-style-type: none"> • Can only analyze and not evaluate the pattern of ginsenosides. • Non-volatile compounds have to be derivatized before being subjected to analysis. 	[3,17, 47,48, 61,62]
Thin Layer Chromatography (TLC)	Can be combined with densitometry technique.	<ul style="list-style-type: none"> • TLC-densitometry is relatively quick and straightforward. • High-performance TLC-densitometry is reproducible, accurate, selective, has low detection limits, and does not require derivatization. 	<ul style="list-style-type: none"> • Less sensitive and selective than MS techniques. • Reagents used in detection of ginsenosides limit reproducibility and accuracy due to their unstable coloration. 	[17,59, 63,64]
IR Spectroscopy - Fourier transformed infrared (FTIR) - Near-infrared (NIR)	IR spectra can undergo mathematical processing, and algorithms can be constructed based on the first derivative, second derivative, and 2D-IR correlation spectra.	<ul style="list-style-type: none"> • FTIR has increased resolution and signal to noise ratio, and decreased size and cost. • NIR is fast, non-destructive, simple to use, and does not produce hazardous waste. 	<ul style="list-style-type: none"> • Calibration in NIR spectroscopy is tedious. • Accuracy and precision in NIR spectroscopy is debatable. 	[17,65-68]
NMR Spectroscopy	Various nuclei such as ^{13}C , ^{19}F , ^{31}P and ^1H have been used in NMR applications. In pulsed NMR spectrometers, a radio frequency pulse is used to excite the nuclei in samples, and information about the number and kinds of protons, as well as their structural arrangement can be obtained.	<ul style="list-style-type: none"> • Sensitive, precise and used universally for qualitative and quantitative analyses. • It is a non-destructive method. 	<ul style="list-style-type: none"> • There is a lack of pure certified reference materials, particularly in the analysis of natural products. 	[69]

Genomic Methods	Methods include restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD), sequence characterized amplified regions (SCAR), genomic libraries, and DNA sequencing techniques.	<ul style="list-style-type: none"> • Prior genomic sequence information is not needed in RAPD and PCR methods. • RAPD is inexpensive, fast and requires small amounts of sample. • AFLP is robust, reliable, has a high resolution and is tolerant to DNA degradation. • RFLP is highly sensitive and produces reproducible results. 	<ul style="list-style-type: none"> • Lack of reproducibility in RAPD. • Prior genetic knowledge of sample is needed in RFLP. • RFLP requires two separate enzymatic steps in the amplification and restriction processes. 	[6,14, 26,54-57,70-76]
Proteomic & Metabolomic Methods	<p>Methods include two-dimensional (2-DE) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), fast protein liquid chromatography (FPLC), tandem MS, DNA microarrays, metabolite profiling and transgenics.</p> <p>Metabolomic studies can be combined with GC, LC, MS, FTIR, Nuclear Magnetic Resonance (NMR), and Raman spectroscopies for analyses.</p>	<ul style="list-style-type: none"> • Information on protein expression and cellular regulation can be obtained. • Enables phenotype to be determined directly. • Transgenics has the potential of improving the production of ginseng plants. 	<ul style="list-style-type: none"> • Does not directly relate mRNA or protein changes to change in biological function. • Lack of availability of the full genome sequence limits proteomic research on ginseng. 	[12,38-40,61, 62,71, 77-80]
Enzyme Immunoassays (EIAs)	Use of monoclonal and polyclonal antibodies, and conjugation with Bovine serum albumin (BSA)	<ul style="list-style-type: none"> • Highly sensitive and specific. 	<ul style="list-style-type: none"> • Presence of other ginsenosides may interfere with results. 	[3,17, 51]

2.2.1. High Performance Liquid Chromatography (HPLC)

HPLC has been the method of choice [8,9,17,19,27,45,63,81] for the analysis of ginsenosides because of its speed, sensitivity and ability to analyze non-volatile, polar compounds [17]. It is commonly achieved by using a reversed phase C₁₈ column, and a

mobile phase gradient of water or phosphate buffers and acetonitrile [5,17,44-46]. Since the ginsenosides are poor chromophores [3,17,52], their peak detection by ultraviolet (UV) rays is limited to short wavelengths at 201-205nm [3,8,27,44,59]. The level of sensitivity for HPLC/UV detection of ginsenosides is not ideal, and many compounds may interfere with the analysis [3,17].

Sample preparations from studies done on HPLC analysis of ginsenosides are as follows: reference ginsenosides are dissolved in methanol to make stock solutions. Standard working solutions are then prepared by diluting an aliquot of the stock solutions with appropriate quantities of methanol. All stock and working solutions are stored at -20°C and brought to room temperature before use [45,52].

Fresh ginseng samples are freeze-dried prior to extraction and analysis [36,81]. The ginsenosides are usually extracted from the dried ginseng material with 80% methanol [5,8,45]. The methanol-water mixtures increase the extractions of ginsenosides as compared to pure methanol [17]. Other common methods include soxhlet [81] and ultrasonic extractions [5,8,17,25,36,45,52,81,82]. Ultrasonic extraction is more popular since a minimum of 20h is required in soxhlet extraction for complete conversion, and hence is not suitable for large quantities of samples [17,81].

Base hydrolysis usually involves adding potassium hydroxide (KOH) to the ginseng extract, neutralizing the resultant solution with potassium hydrogen phosphate (KH_2PO_4), then making up to volume with acetonitrile [5,8,81].

Studies have stated that storage of dried ginseng roots and its powder at low temperatures of $5\pm 2^\circ\text{C}$ can last for approximately three months due to negligible loss of

ginsenosides [8,42]. However, it is also recommended that such herbal products be stored in packaging which protects them from light and atmospheric moisture (<10%) [8].

Other interfaces used for detection of ginsenosides include an evaporative light scattering detector (ELSD) [17,45], fluorometric [3,27] or diode array detection (DAD) [44,63,83] methods. The advantages of HPLC/ELSD are many. It is more sensitive than the HPLC/UV method, and can quantify the many types of ginsenosides [3,27,45]. It is also fast, relatively cheap and straight-forward. HPLC combined with fluorescence detection is not only reliable and sensitive, but it can be used to detect all ginsenosides and their metabolites which have the C₂₄-C₂₅ double bond as well [3,17].

For the qualitative analysis of *Panax ginseng*, HPLC has been combined with thermospray (TSP) and frit-fast atom bombardment (Frit-FAB) interfaces [17,27], but due to its poor reproducibility and lack of ability to detect the thermolabile malonyl-ginsenosides, other techniques have been employed instead.

Certain issues have been discussed in various studies regarding the HPLC analysis of ginsenosides. The importance of the malonyl-ginsenosides (mRb₁, mRb₂, mRc, mRd) must be recognized due to their potential to undergo demalonylation by heat and hydrolysis [5,8,27,81]. This could affect the concentration of total ginsenosides since the malonyl-ginsenosides are present in significant amounts [5,81], and hence the quality of ginseng samples. For example, red ginseng is processed by steaming and thus has a lack of the malonyl-ginsenosides due to the heat as compared to white ginseng [17]. However, this problem can be overcome by basic hydrolysis of the residue with 5% potassium hydroxide (KOH) solution to convert the malonyl-ginsenosides to their respective neutral ginsenosides which are more readily determined by HPLC [5,8,81]. However, this is only

an estimate as other derivatives, such as small concentrations of Rb₁ acetate, have been reported in *Panax quinquefolium* [81].

As there are no standards to the malonyl-ginsenosides, indirect methods have to be used to detect their presence. An indirect method of HPLC analysis was done in a study [8] in which each sample of ginseng was analyzed twice. The first analysis was to quantify the amount of neutral ginsenosides in the extract, after which the extract was hydrolyzed and then analyzed a second time. The purpose of the hydrolysis process was to convert the malonyl ginsenosides to their respective neutral ginsenosides. Both chromatograms of the original and hydrolyzed extracts were compared, and the concentration of malonyl ginsenosides in the original extract was calculated.

Another problem is the use of methanol for the alcoholic extraction of ginsenosides. Due to its toxic properties, methanol is not acceptable for food processing [8]. Ethanol can be used as an alternative, but is not as efficient as methanol [8,83]. In a study by Du et al. [5], it is shown that the neutral ginsenosides were best extracted with 70% ethanol, as compared to only 40% for the malonyl ginsenosides. This results in maximum extraction of the total ginsenosides with 60% ethanol.

2.2.2. Mass Spectrometry (MS)

Field desorption, secondary ion and electron impact mass spectrometry are all techniques that are available in the past. These methods require chemical derivatization before analysis, and hence are time consuming processes [20,60]. However, in recent years, various mass spectrometric techniques have been used in combination with chromatography techniques to characterize ginseng components [47]. The most promising

method for analysis of ginsenosides seems to be the use of HPLC in conjunction with mass spectrometry (MS or MS/MS) [17,27,44,47,50]. In terms of sensitivity and specificity, an MS detector is better than that of UV or ELSD detection [44,45,50]. This is because MS can be used for confirmatory identification of the ginsenoside peaks and the results are not ambiguous [44]. Among the various methods, the LC-tandem mass spectrometry (LC/MS/MS) technique is to date is the most sensitive method for quantification of ginsenosides [3,25]. The electrospray ionization mass spectrometry (ESI-MS) [20,27,60,83] method has been effective in the structural characterization of various polar and thermolabile substances such as polypeptides and carbohydrates, and it avoids tedious derivatization and separation steps [60]. It is also highly sensitive, has a better reproducibility than other kinds of ionization [17,60], and is considered to couple with HPLC the best [50] as it can overcome problems associated with the thermolabile malonyl-ginsenosides [17,27] and low molecular ion abundance levels [47]. Studies also show that when combined with the collision-induced dissociation (CID) methodology, structural information about molecular weight, type of aglycone, and masses and sequences of sugar residues of the ginsenosides can also be determined [17,20]. However, this technique has been claimed to be too costly to be used for routine analyses in certain studies [27,45].

2.2.3. Gas Chromatography (GC) and Thin-layer Chromatography (TLC)

Thin-layer chromatography (TLC) [17,59,63,64] and gas chromatography (GC) [3,17,47,63] are also commonly used in the analysis of ginsenosides. Although TLC has also been used to detect ginsenosides, its sensitivity and selectivity in identification is

lower than that of LC/MS or LC/MS/MS, thus limiting its reproducibility [3,11,45]. Several studies [17,64] have indicated that the TLC detection of ginsenosides is usually achieved by using sulphuric acid alone or its mixtures with aromatic aldehydes like vanillin or anisaldehyde. However, these reagents limit the reproducibility and accuracy of the TLC method due to their unstable coloration. In contrast, densitometric determination of ginsenosides by high-performance thin-layer chromatography (HPTLC) offers the advantages of being reproducible, accurate, selective, having low detection limits and it does not require a derivatization step [17,64]. GC, on the other hand, does give good sensitivity and resolution as well, but it can only analyze and not evaluate the complex pattern of the protopanaxadiol and protopanaxatriol ginsenosides [3,17]. Furthermore, the ginsenosides have to be trimethylsilyl (TMS)-derivatized and hydrolyzed [17,59,84] in GC, unlike TLC-densitometry which is relatively quick and straightforward [59].

2.2.4. Enzyme Immunoassay (EIA)

Enzyme immunoassays (EIAs) for the ginsenosides F₁, Rb₁, Rf and Rg₁ have been developed [51] and are highly sensitive, but they are also specific, hence they are only suitable for determining one ginsenoside, as the presence of other co-existing ginsenosides may interfere with the results [3,17]. A study of an enzyme-linked immunosorbent assay (ELISA) for panaxytriol [85] has been described by Saita et al. The described assay is able to detect other polyacetylenic compounds as shown by the antibody cross-reactions. The authors postulate that the specificity of the ELISA is toward the glycol moiety of the panaxytriol, thus there will be a cross-reaction to polyacetylenic

alcohols with this glycol moiety. According to another study [51], bovine serum albumin (BSA) can be combined with the ginsenoside F_1 through its glucose moiety at C_{20} so that it can be used as an immunogen. As the protopanaxatriols can be differentiated from the protopanaxadiols by their α -hydroxyl group at the C_6 position, rabbits which are inoculated with the ginsenoside F_1 -BSA antigen can produce antibodies that can detect the protopanaxatriols. These antibodies have higher affinities for ginsenoside F_1 than the protopanaxadiols and other ginsenosides that have carbohydrate moieties attached at the C_3 or C_6 positions.

2.2.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy has been used as a qualitative and quantitative tool for elucidation of structures. A recent review by Pauli et al. [69] describes the advancement of NMR, in particular quantitative NMR (qNMR), in the area of natural products research. The aim of the review is to demonstrate that this technology is able to provide access to both definitive chemical structures and the “singleton character” of single chemical entities (SCEs).

The parameters which define the NMR methodology include chemical shift, spin-spin and dipolar coupling, through-space interactions and relaxation [69]. Although various nuclei such as ^{13}C , ^{19}F and ^{31}P have been used in NMR applications, proton NMR (^1H NMR) is more commonly used for quantitative analyses of natural products as proton spectra are considered rapid, and able to accurately characterize sample compositions under proper conditions [69].

The underlying physicochemical mechanism of qNMR is different from chromatography techniques used in sample quantitation [69]. The principle of NMR spectrometers is based on pulsed NMR techniques [69], in which a radio frequency pulse is used to excite the nuclei in the sample. The ratio of the number of protons in a molecule can be determined, since the number of nuclei is proportional to the integrated intensity of the NMR signal. Information about the kinds of protons and their structural arrangement can also be obtained from observations of the spin-spin coupling patterns and chemical shift positions of the proton absorptions.

TCMs have different chemical constituents in which characteristic NMR fingerprints can be assigned [86]. As such, NMR methodologies have been used for the analysis of herbs such as ginseng [86], kava-kava [87], St. John's wort [88], and arnica [89]. However, this technique is most popular in the analysis of ginkgo for the quantitation of bilobalide and ginkgolides [69]. ^1H NMR spectroscopy can also be used for analyzing enantiomeric compositions, as well as evaluate active pharmaceutical ingredients and stability testing of natural product preparations [69].

Qin and Zhao [86] have recently demonstrated that ginseng species can be differentiated from other TCMs based on their ^1H NMR fingerprints. They postulate that the peaks in the region of δ 4.88-6.25 are similar among *Panax ginseng*, *Panax quinquefolius* and *Panax notoginseng*. Furthermore, the signals at δ 0.87-1.64 and δ 4.16-5.12 are due to ginsenosides – the active constituents of ginseng. Ginsenoside Rg₃ has a chiral centre at carbon-20 [90]. This gives rise to enantiomers which exhibit different stereoselectivities in the regulation of voltage-dependent Na⁺ channel activity [90]. Using a combination of NMR spectroscopy and molecular dynamics simulations, Kang et al.

[90] was able to conclude that the 20(S)-Rg₃ inhibits Na⁺ channel currents, but not the 20(R)-Rg₃. In another study by Luchtefeld et al. [91], it was also found that ¹³C NMR can be used to identify complex carbohydrates present in the various dosage forms of ginseng.

Thus, in comparison to traditional chromatography techniques, NMR spectroscopy offers the advantages [69] of sensitivity, precision and universality. In addition, it is a non-destructive method, and can be used as a replacement of other analytical techniques [69]. However, the lack of pure certified reference materials limits its use, particularly in the analysis of natural products [69].

2.2.6. Genomics and Proteomics

Although there has been considerable commercial interest in ginsenosides, unfortunately little is known about their genes and biosynthesis pathways [70,71]. As the authentic and adulterant materials have different DNA sequences, these cheaper or less effective substitutes can be distinguished based on various nucleic polymorphisms [6]. Hence, genetic and genomic methods have, in recent years, become popular in analyzing ginseng populations. However, limitations of these techniques are that they are time-consuming, and when there is insufficient amount or degradation of template DNA in ginseng dried extracts and softgels [11].

Restriction fragment length polymorphism (RFLP) [54], microsatellite-primed (MP-PCR) [72] and arbitrarily primed polymerase chain reactions (AP-PCR) [14,57], randomly amplified polymorphic DNA (RAPD) [14,26,54,57] and sequence characterized amplified regions (SCAR) [6,73] analyses are examples of such methods. No prior genomic sequence information is needed in RAPD and AP-PCR methods [72] as the

results are not affected by the source or age of materials [14]. RAPD uses primers which have less nucleotides than AP-PCR [14]. Furthermore, RAPD offers other advantages such as low cost, speed and the use of small amounts of plant material when compared with isozyme and RFLP techniques [54]. However, its lack of reproducibility limits its use [54,57]. On the other hand, SCAR analysis can indicate a 25bp insertion in the sequence of *P. quinquefolius*, hence distinguishing it from *P. ginseng* [73]. The use of expressed sequence tags (ESTs) [70,71] and bacterial artificial chromosome (BAC) sequences [71,74] in DNA sequencing techniques also help in identifying the genes involved in ginsenoside secondary metabolite biosynthesis.

Utilization of genomic libraries is common in techniques that require cloning of repetitive DNA sequences. These clones can be used as DNA probes [57,92] to distinguish *P. ginseng* and *P. quinquefolius* in DNA fingerprinting. Examples of such clones include: del-like retrotransposon sequences, plant telomeric repeats (TTTAGGG), “*P. ginseng*-specific” 170-bp satellite repeats [57], low C_0t DNA [72,92], as well as nuclear ribosomal internal transcribed spacer (ITS) regions [26,55,56].

Amplified (AFLP) and restriction fragment length polymorphisms (RFLP) have been used in various studies [55,56,72,75,76] to determine the genetic fingerprints of ginseng and other Chinese medical herbs. These techniques involve the digestion of the template DNAs and their ligation to primers. Then by adding or removing nucleotides at the 3' end of the primers, different sets of restriction fragments of genomic DNA can be PCR amplified. This will determine the complexity level of the AFLP and RFLP profiles [56,75,76]. In comparison with traditional methods of authentication, RFLP offers the advantages of being highly sensitive, requiring only micrograms of the sample, and

capable of producing reliable and reproducible results since they are not affected by the physical conditions of the herb sample [56]. However, its disadvantages include the need for two separate enzymatic steps in the amplification and restriction processes, and prior genetic knowledge of the sample [6]. On the other hand, AFLP is not only robust and reliable when compared to RAPD and AP-PCR [76], it has a high resolution and is tolerant to DNA degradation as well [75]. In one of the studies [76], direct amplification of minisatellite region DNA (DAMD) has also been successfully carried out on a minisatellite DNA, which contains eight repeats of 5'-AGGACTCATCACATTGTTACTC, to differentiate between *P. ginseng* and *P. quinquefolius*.

Technical improvements in recent years have made proteomics an essential technology to bridge the gap between genotype and phenotype in the field of biological sciences [61,71]. Information on protein expression and cellular regulation can be obtained through protein identification in proteomic analysis [71,77]. Methods used in proteomic research of *P. ginseng* include two-dimensional (2-DE) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [12,71,77,78], fast protein liquid chromatography (FPLC) [78], tandem MS [71,77], DNA microarray [62,71], metabolite profiling [61,62,71,79] and production of transgenic plants [38-40,71,80]. In a study by Lum et al. [12], he demonstrated that 2-DE can be used to discriminate different species and parts of ginseng. However, proteomic analysis of plant samples, particularly ginseng, is difficult due to the lack of genomic DNA sequence information in ginseng populations [71]. It has been suggested that proteins can be identified through the use of EST

databases, but their use is limited by significant error rates in the EST sequences and the lack of protein representations [71].

Metabolomics is an emerging field complementary to both transcriptomics and proteomics [61,79]. Factors influencing its robustness include its speed, sensitivity and reliability, ability to handle large numbers of metabolites, as well as its suitability for automation [79]. Although MS and molecular spectroscopy are used commonly to analyze metabolites, GC and LC technologies can be coupled to improve their sensitivity, selectivity and resolution [61]. Metabolic fingerprinting is fast, and is preferred for rapid characterizations of metabolites if the changes in the metabolome are reproducible. MS, NMR, FTIR and Raman spectroscopies can be used to analyze large quantities of crude extracts or whole cells via their mass-to-charge (m/z) ratios, chemical shifts or wavenumbers [61].

2.2.7. Other Methods

Other analytical techniques include ion chromatography separation with pulsed amperometric detection (IC-PAD), which has high sensitivity for detection of ginsenosides [3]. In a recent local study, open column chromatography was used for the identification of saponins from *P. notoginseng* [44]. Colorimetry has also been used to determine the amount of total ginsenosides [59]. This non-specific method is based on the reactions of the ginsenoside triterpene skeleton. Micellar electrokinetic chromatography (MEKC) has been employed as an alternative method to capillary electrophoresis (CE) since it is not applicable for ginsenosides analysis due to the absence of charge in ginsenosides [17].

2.3. Infrared (IR) Spectroscopy

As IR spectroscopy will form the groundwork of the current research, a section has been dedicated to the physicochemical basis and applications behind this analysis technique.

Figure 2.3. Electromagnetic spectrum showing the various bands of the infrared region.

2.3.1. Physicochemical Basis

The infrared region of the electromagnetic spectrum (Figure 2.3) lies between the wavelengths of visible light and microwaves. For convenience, this region is further defined as the near infrared (NIR), mid-infrared (MIR) and the far infrared (FIR) bands. The NIR region extends from 12800 – 4000cm⁻¹, while the MIR and FIR regions are from 4000 – 250cm⁻¹ and 250 – 10cm⁻¹ respectively [93]. The absorption positions can be presented as wavelengths (λ) in μ m or as wavenumbers ($\tilde{\nu}$). Wavenumbers are expressed as the reciprocal of wavelengths in cm⁻¹ [94], and can be interconverted by the following equation [95]:

$$\tilde{\nu} = \frac{1}{\lambda} \times 10^4 \quad (2.1)$$

In contrast, wavenumbers are directly proportional to both the frequency and energy of the absorption [95]. Hence a higher wavenumber will correspond to a higher energy. This relationship is defined by [94]:

$$E = h\nu, \text{ where } h = \text{Planck's constant} \quad (2.2)$$

Any organic molecule having covalent bonds will absorb infrared radiation, resulting in it being excited to a higher energy state. The energy absorbed will cause an increase in the amplitude of the vibrational frequencies of the bonds. However, only bonds which have a net change in dipole moment as a function of time are able to absorb infrared radiation [94,96]. These vibrational motions can be categorized into stretching and bending modes [94]. Figure 2.4 shows the normal vibrational modes of a molecule, using the methylene group as an example. Stretching vibrations usually occur at higher frequencies as compared to bending vibrations. There are two modes of stretching: symmetric and asymmetric, and the symmetric stretch vibrations occur at lower frequencies than asymmetric stretch vibrations. Bending vibrations, on the other hand, can be described as in-plane or out-of-plane bending, within which scissoring, rocking, wagging and twisting are the more common modes.

Figure 2.4. Vibrational modes of stretching and bending of a -CH_2 group.

The principle in which IR spectroscopy is based upon [65] is when low energy transitions occur in molecules of the sample material, they absorb the IR radiation, hence resulting in absorbance bands which can be used for quantitative analysis or identification of the major structural features or basic functional groups of the molecules. No two chemical structures will have identical IR spectra since each type of bond in different compounds will have different vibrational frequencies [94]. Hence the IR spectra, which is a usually plot of percent transmittance or absorbance versus frequency expressed in wavenumbers ($1/\lambda$ or cm^{-1}) [65,95], can be used as a “fingerprint” for compound identification [94]. Since absorbance is inversely proportional to transmittance (T), and

transmittance is the ratio of light transmitted by the sample (I) to incident light on the sample (I_0), their relationship can be represented by [95]:

$$A = \log_{10} \left(\frac{1}{T} \right) = -\log_{10} T = -\log_{10} \frac{I}{I_0} \quad (2.3)$$

Analysis of an IR spectra is based on Beer-Lambert's Law [65,66,97], which is the relationship between the amount of light absorbed by a certain species and its concentration. It is defined as:

$$A = -\log_{10} \left(\frac{T}{100} \right) = \varepsilon b C \quad (2.4)$$

where, A is the absorbance of a solution, $T(\%)$ is the percent transmittance, ε is the molar absorptivity in $\text{mol}^{-1}\text{cm}^{-1}$, b is the pathlength expressed in cm, and C is the molar concentration of the absorbing solute. A system that follows Beer's law will show linearity when a graph of absorbance against concentration is plotted [66].

With advancement in computing technology, IR spectra can now undergo complex mathematical processing which can reveal hidden details [65]. As spectra are subjected to scattering effects, algorithms have been introduced so that linear calibration models can be constructed [67]. These algorithms are based on the use of first derivative IR spectra, secondary derivative spectra, and 2D-IR correlation spectra [65,67]. The derivative spectra emphasize the rates of change in the recorded data that are difficult to detect from the zeroth-order spectrum [98]. The first derivative spectrum is the rate of change across a

scan and it reflects points of maximum change in the original spectrum; while the second derivative shows the change in the first derivative and allows determination of the exact peak locations [65]. Second derivatives are usually employed for peak picking since both baseline offset and slope are removed from the spectral data, unlike its first derivative counterpart in which only the offset is removed [99]. A negative peak in a second derivative corresponds to a wavenumber of maximum absorbance of the band in the original spectrum [98-100]. Besides removing baseline drift [101], it can also be used to suppress scattering in measurements [67] and thus, acts as a good noise filter for the spectra [102]. As a result, tiny differences in the IR spectrum can be amplified via their derivative spectra and this is especially useful when the peaks of overlapping bands need to be separated [102]. The 2D-IR correlation analysis technique increases the resolution of the IR spectra, so that information about the molecular structure of the sample can be obtained [43].

2.3.2. Fourier Transformed Infrared (FTIR) Spectroscopy

Fourier transformed spectrometers are based on [95] interferometers which divide and recombine radiant beams to produce repetitive interference signals based on their optical path difference. The Michelson interferometer is the most common, and it consists of a beamsplitter, and a fixed mirror which is perpendicular to another moving mirror (Figure 2.5). The IR beam is directed into the interferometer where half of it is transmitted to the fixed mirror, and the other half to the moving mirror. They then recombine at the beamsplitter. An interference pattern is generated in response to the change in relative

position of the moving mirror to the fixed mirror, and the resultant beam is then passed through the sample and detected.

Figure 2.5. Principle of a Michelson interferometer. Adapted from reference [95].

Since the interferogram is a time-domain spectrum displaying an intensity versus time plot, a Fourier transformation mathematical operation is needed to separate the individual frequencies from the interferogram and convert them into the final frequency domain IR spectrum which displays a plot of intensity versus frequency [94,95]. Fourier transform is a sum of sinusoidal functions that is represented by a function $f(t)$ in relation to angular frequencies (ω) [103-106]. The forward and backward transform equations are shown below:

$$F(\omega) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} f(t) e^{-i\omega t} dt \quad (2.5)$$

$$f(t) = F^{-1}(F)(t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} F(\omega) e^{i\omega t} d\omega \quad (2.6)$$

As the FTIR spectrometer can rapidly acquire an interferogram, Fourier transformation can be performed on cumulative interferograms giving a spectrum with better signal-to-noise ratio, thus providing a spectrometer with higher sensitivity and speed [94].

Fourier transformed infrared (FTIR) techniques have recently been used for analyses of solid and liquid samples [66], and is popular for analyzing protein structures in biological studies because information on the secondary structure of proteins is reflected by the amide bands in the spectra [107]. Other advantages, besides increased resolution and signal-to-noise ratio, are its decreased size and cost [65]. Both qualitative and quantitative analyses can be performed using the mid-infrared spectrum as the spectral data provides information about the chemical species within a particular sample based on their vibrational transitions [66].

2.3.3. Near-infrared (NIR) Spectroscopy

NIR spectroscopy is a technique based on absorption and reflectance of monochromatic radiation by samples over a wavelength range of 25000-4000cm⁻¹ [17,18]. The NIR spectra of organic molecules are primarily due to overtone and combination bands of C-H, N-H, O-H and C=O groups [93,108,109]. The overtones are anharmonic and the spectra are complex due to the result of many overlapping peaks and valleys, thus they appear to be poorly resolved, broader, and are not directly interpretable as compared to other spectral regions [93,108,109]. However, much information can be obtained from this spectral region [93] since the overtones and stretch combination bands of C-H and O-

H occur four times and three times respectively. Furthermore, the N-H overtones also occur three times in this region.

NIR spectroscopy has been successfully employed in the food and pharmaceutical industries [17,18,65,67,68,101]. Examples include identification and analysis of active ingredients in various dosage forms, determining pharmaceutical powder homogeneity and quality of raw materials due to their distinct physical properties (such as particle size distribution and compactness), protein analysis and other clinical applications [65,67]. NIR reflectance spectroscopy can also be used to differentiate various herbal products via pattern recognitions [101]. This technique is fast, non-destructive, does not produce any hazardous waste, and is simple to use when compared to HPLC [17,18,43,67,68,101,108,110]. The biggest advantage is that real-time data can be obtained, and little or no sample preparation is needed [108]. The drawback, however, is that it is not a stand-alone technology [108] – its calibration is tedious as it requires several natural samples with known analyte concentrations that are obtained by suitable reference methods [17,67,68]. It is difficult to find specific absorption bands in NIR spectra as these bands combine the fundamental vibrations of the –CH, –NH, –OH and –C=O groups from the MIR range [101,108]. Its accuracy and precision is debatable with some studies stating that it is less precise than HPLC methods [18], while others state that its results are comparable to those obtained with HPLC [17].

2.4. Biosensors

Biosensor construction and application has previously been described by several authors [111-114], and is summarized in Figure 2.6. Essentially a self-contained integrated

device, a biosensor consists of a biological recognition element that is in direct contact with a transducer [113,115,116]. Various methods such as antigen-antibody reactions, immobilized biological components like bacteria and enzymes, and nucleic acid probes [1,97,114-117] have been employed in biosensor technology. Common immobilization methods include physical entrapment, encapsulation, covalent binding to a surface, cross-linking between molecules, and surface adsorption [113,114,116,118]. Transducers that are coupled onto the biosensor devices can be based on electrochemistry (hydrogen peroxide, oxygen, electron transfer; using electrodes), mass change (piezoelectric crystals or surface acoustic wave devices), optics (absorbance, fluorescence, chemiluminescence; using optrodes), or temperature (thermistors or heat-sensitive sensors) [97,114-117]. These transducers convert a particular biochemical event into electrical signals which can be monitored by amperometric, potentiometric or conductimetric means [117]. The signals are then analyzed by a computer.

An ideal biosensor should not only be accurate and reliable, but also robust, tolerant to processing conditions, and capable of monitoring simultaneous multianalytes [97]. It should also be cheap, easy to install and maintain [1,118]. Biosensors have been used for environmental [97,116] and bioprocess monitoring [97,119,120], in the seafood industry [117], food technology [97,114,118,121], and even in military defense [97]. The most successful biosensors which are currently available commercially in the healthcare industry are glucose sensors used for diabetic patients [97,115-117]. Other biosensors include those for drugs (digoxin, phenytoin, phenobarbital, theophylline, gemfibrozil) [67,115,122], blood lactate, cholesterol, aniline, pyruvate, glutamine, glutamate and possibly even creatinine and urea analyses [115].

Figure 2.6. Principles and components in biosensor technology.

With the advances in optical fibers and laser technology, particular attention has been directed at optical biosensors which are based on methods like absorption of UV or visible light, luminescence, fluorescence, phosphorescence, reflectance, refraction, light scattering, surface plasmon resonance (SPR) and evanescent wave sensing [97,114,115,123]. A fiber optic system usually consists of an appropriate light detector, an optical interface, input and output fiber optic cables, and a sensing device [66]. These sensors operate on a common principle in which light that enters an optical device is directed toward a sensing surface and reflected out again. By monitoring this reflected

light using a detector, information about physical events occurring at the sensing surface can be obtained [115]. The source of light is usually placed close to, or optically concentrated at the fiber end [66]. Sensor technology is based on attenuated total reflection (ATR), specular reflection, reflection-absorption and diffuse reflection principles [65,66]. The cladding or jacket of an optic fiber can be removed to produce changes in the refractive index of the outside material, and hence the evanescent field [123]. A major advantage is that no separation step of the free and bound labels is required due to the lack of need of labeling [114,115]. Other advantages [97,123] include being small and lightweight, yet sensitive. It also encompasses a large bandwidth and is immune to electromagnetic interference (EMI).

The materials used to make infrared fibers include chalcogenide glasses, heavy metal fluorides and silver halides [66]. The spectral ranges of chalcogenide glass, fluoride glass, sapphire and silver bromide/ chloride (AgBr/ AgCl) IR fibers are $4000\text{-}900\text{cm}^{-1}$, $20000\text{-}2325\text{cm}^{-1}$, $50000\text{-}2500\text{cm}^{-1}$ and $3000\text{-}667\text{cm}^{-1}$ respectively [65,66]. Among the four types of fibers mentioned, chalcogenide glass has been most commonly used for the mid-infrared spectrum ($4000\text{-}400\text{cm}^{-1}$) due to its wide spectral range, and is formed from Group 5 elements like arsenic, germanium and antimony, combined with the heavier Group 6 elements like sulfur, selenium or tellurium [66]. A disadvantage is the high toxicity of arsenic in its chemical composition, thus AgBr/ AgCl fibers have been used in more recent years due to its lack of toxicity, but yet has the appropriate spectral range to be used for in-vivo applications [65].

Sensitivity and energy throughput can be maximized by aligning an optic fiber with a deuterium triglyceride (DTGS) or mercury cadmium telluride (MCT) detector

[65,66]. The DTGS detector usually comes with the standard instrument due to its relatively low cost, but the MCT detector is more sensitive [65].

Several factors have to be considered in the design of the fiber optic sensors [65,66]. It must provide sufficient sensitivity and pathlength, as well as an optimized signal-to-noise ratio for analysis. In addition, the sensor must also be inexpensive, robust and easy to use. It should be flexible enough to utilize a variety of sensor technologies for sampling, yet remain inert during analysis and so that it can be used in almost any kind of analytical environment. The sensor should be easy to clean so that the risk of sample cross-contamination is reduced, and its results must be quantitatively reproducible. If the sensor is to be used for in-vivo studies, it must be non-toxic as well. For fiber optic systems to cater for commercial uses, detecting and signal-processing technologies should be included so that the systems are complete [123]. This is because users typically look for sensor systems whose prices can match their performances.

In comparison with chromatographic methods, the two major advantages which biosensors have are their high selectivity and rapid response [114,118,121]. Furthermore, the use of fiber optics eliminates the need for sample manipulations due to its ability to provide direct measurements, hence it is appealing in the analysis of powders or granulate samples [67]. There have been reports stating that information can be obtained from a sample without removing it from the packaging, even glass or plastic, when using near-infrared fiber optics [66]. Furthermore, the chemical compositions of both sample and packaging can also be obtained with mid-infrared fiber optics [66].

2.5. Summary

The use of biosensors in the area of complementary and alternative medicine is definitely wide ranging. A microphysiometer based on a multi-light addressable potentiometric sensor (MLAPS) has been developed to observe the influences of some Chinese medicines (medlar, hemlock parsley, scutellaria) [122]. Other possible applications include those for biophysical cancer treatment [124], evaluating the radical scavenging effects of various fruits and vegetables [125], determining the effect of garlic on arteriosclerotic plaque formation [126], detecting the needle force during the acupuncture process [127], and even as a possible tool on the human laying of hands in patient-healer relationships [128]. A superoxide dismutase (SOD) biosensor was developed by Campanella et al. [129] to evaluate the antioxidant capacity of ginseng tea. Baulsir and Simler [66] had also previously postulated that by combining the sensitivity and high throughput of FTIR spectrometers with high optical efficiency mid-IR fiber optic materials, an optimized tool for analysis of solid and liquid samples could be developed. However, despite its potential, there is currently no commercially available instrumentation to perform rapid ginseng analysis. Therefore, this work was done with the aim of developing a novel sensing strategy based on western analytical techniques to perform rapid analysis of ginseng products.

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1. Material

3.1.1. Ginsenoside Standards

As the active constituents of ginseng are its ginsenosides, pure ginsenoside standards were obtained to test if they could be differentiated based on their IR spectra. Four types of ginsenoside standards were obtained from Eu Yan Sang International Ltd. – ginsenosides Rb₁, Re, Rg₁, and F₁₁, while ginsenoside Rc was purchased from Sigma-Aldrich Inc. (Asia-Pacific, Singapore).

3.1.2. Ginseng Roots

Two kinds of ginseng were obtained locally and from overseas: Asian ginseng (人參) and American ginseng (西洋參, 泡參). The ginsengs were classified by the suppliers into various grades which were indicative of their quality. A common trend was noticed with regards to the ginseng grades – a better quality ginseng would be given a higher grade and was more expensive. However, the classification of the quality grades varied with different suppliers.

Six grades of American ginseng (grades 1 to 6) and three grades of Asian ginseng roots (grades 5 to 7) were purchased from a branch of the major Chinese medicinal chain (Hockhua Ginseng Birdnest Trading Enterprises) in Singapore. Additional Asian ginseng root samples were obtained and these included a fresh Asian ginseng root sample purchased from a local supermarket (grade 1), a box of dried Asian ginseng root pieces purchased from the same local medicinal hall (grade 2), and two boxes of dried root

samples from gift hampers (grades 3 and 4). To ensure consistency, these grades were also allocated by the same retailer in the local medicinal hall based on his experience in the morphology of the ginseng roots, with grades 1 and 4 having the lowest and highest costs respectively. Three grades of ginseng root powders (grades 2, 5 and 10) were also purchased from a medicinal shop (Da Xin Hang 大新行) in Sheung Wan (上環), Hong Kong, to further establish the analytical strategy used in this study. Thus, the identity of these ginsengs was not made known at the point of purchase.

3.1.3. Adulterants

Two morphological adulterants were obtained to test the feasibility of distinguishing them from the authentic ginseng species. *Platycodon grandiflorum*, also known as Jiegeng (桔梗), was purchased from the same local medicinal hall in Singapore, while sawdust samples were obtained from a technical workshop in a local institution.

3.1.4. Commercial Products

Three commercial products which claimed in their labels to contain pure ginsengs were obtained to compare the raw ginseng roots with finished products. American ginseng tea was purchased from a separate local medicinal shop, and bottles of American and Korean capsules were purchased from a branch of a major health supplement chain in Singapore.

3.2. Sample Preparation and Testing

The American and Asian ginseng root samples, as well as jiepeng samples, were all cut into small pieces and ground into fine powder before analysis. The three grades of ginsengs purchased from Hong Kong were stored separately in packaging which protected them from light and moisture before being air-flown back to Singapore, and the fresh Asian ginseng root purchased from the supermarket was dried under the sun till it hardened before being ground into powder. All ginseng and jiepeng samples were stored at 4-5°C and brought to room temperature before use.

Fifty samples of sawdust, American and Asian ginsengs from Singapore, ten samples of jiepeng and of each grade of ginseng from Hong Kong, as well as three samples of each ginsenoside, were used for analysis. Each sample was mixed uniformly with 100mg of spectroscopic grade potassium bromide (KBr) powder (1% w/w) using an agate pestle and mortar, and then pressed into a pellet.

Twenty samples of each commercial product were also analyzed using the same processing technique. The tea leaves were taken out from the American ginseng tea bags and ground into powder form, while the American and Korean ginseng powder were taken out of their capsule shells before mixing with the KBr powder.

The IR spectra were recorded in the mid-IR region of 4000 – 400cm⁻¹ and near-IR region of 7800 – 4000cm⁻¹ on a Shimadzu IRPrestige-21 FTIR spectrometer (Shimadzu Corporation Pte. Ltd., Asia Pacific), whose working principle is based on the Michelson interferometer as previously described in Figure 2.5, equipped with a KBr beamsplitter and a deuterated L-alanine triglycine sulfide (DLATGS) detector. A “tablet holder” of 13mm diameter located in the centre of the beam was used to hold the pellet samples.

Each spectrum was an average of 40 scans co-added at 4cm^{-1} resolution. Pure KBr background spectra were recorded before analysis of the samples.

3.3. Data Analysis

3.3.1. General Spectra

Prior to data analysis, each spectrum was baseline corrected with their absorbance normalized with the Shimadzu IRsolution 1.10[®] software program (Creon Lab Control AG, Shimadzu Corporation Pte. Ltd., Asia Pacific), so that the peak absorbance of the most intense band was set to unity. The corrected spectra were then analyzed for pattern similarity and functional group identification, then transferred into the statistical software program The Unscrambler[®] 9.2 (CAMO Software India Pvt. Ltd., Bangalore, India) for statistical analysis.

3.3.2. Second-derivative Spectra

The second derivative spectrum is the rate of change of the slope of the spectrum tangent, and is derived by replacing the first derivative reflectance value at a particular wavelength by the difference of reflectance at the nearest wavelengths [109]. It allows the distinguishing of overlapping peaks, as well as removal of linear background from the raw spectra [102]. The most common method for spectral smoothing and differentiation is the Savitzky and Golay algorithm [130-134] which is a moving window averaging method. The random noise in the spectral signal can be reduced by fitting data for small wavelength intervals by a polynomial of adequate degree so that the fitted values are a better estimate than the measured values [135]. Considering a set of $2m + 1$ consecutive

points ranging from $i = -m$ to $i = +m$ and a midpoint of $i = 0$, the evaluation of the s th derivative at the central point (a_{nsm}) based on an n th degree polynomial fit, is given by the formula:

$$a_{nsm} = \frac{\sum_{i=-m}^{i=m} c_{ism} y_i}{\Delta x^s N_{sm}} \quad (3.1)$$

where, s is the s th derivative, c_{ism} is the convolution weight of the i th point, y_i its value, N is the number of convoluting integers, and Δx is the sampling interval [130]. Hence by using a quadratic polynomial and a five-point moving window, the first derivative is given by:

$$\frac{dy}{d\lambda} = \frac{1}{10\Delta\lambda} (-2y_{i-2} - y_{i-1} + y_{i+1} + 2y_{i+2}) \quad (3.2)$$

and the second derivative is given by:

$$\frac{d^2y}{d\lambda^2} = \frac{1}{7\Delta\lambda^2} (2y_{i-2} - y_{i-1} - 2y_i - y_{i+1} + 2y_{i+2}) \quad (3.3)$$

where, λ represents the wavelength and y represents the absorbance or spectral intensity [98].

The original IR spectra were converted to their corresponding second derivatives with the same Shimadzu IRsolution 1.10[®] software program (Creon Lab Control AG,

Shimadzu Corporation Pte. Ltd., Asia Pacific) using the 23-point and 45-point Savitzky and Golay algorithms for the MIR and NIR spectra respectively. They were also transferred to The Unscrambler[®] 9.2 (CAMO Software India Pvt. Ltd., Bangalore, India) for statistical analysis.

3.3.3. Principal Component Analysis (PCA)

Principal component analysis or PCA, a type of chemometric analysis technique, was performed on the original and second derivative spectra as a tool for data visualization. It was done with the help of The Unscrambler[®] 9.2, from CAMO software India Pvt. Ltd. (Bangalore, India, Asia-Pacific), into which the MIR and NIR spectra were directly imported.

PCA is a mathematical projection method that is widely used for reorganizing information [136] and explaining causes of variance in a set of data [109], in this case – IR spectra. It becomes useful when dealing with spectroscopic data because there are a large number of variables [136]. The goals of PCA are thus to simplify and enable data reduction, find classes, similarities or relationships among the variables, as well as to detect outliers [137].

The principle of PCA [109,135,136] is to “discover” new variables known as “Principal Components (PCs)” which contain most of the variability of the data set, thus enabling the visualization of information with considerably lower variables than the original data. The PCs are ranked in order of variance. The first PC is the direction through the data which explains the most variation in the data, while the second PC is orthogonal to the first and describes the maximum amount of variability that is remaining.

This goes on until the last PCs which might be irrelevant variance or noise, and hence can be eliminated without losing important data information. Multiplying the PCs by coefficients will produce new values known as “scores” which describe the PCs, and the signs of the scores do not hold any meaning in PC space. The choice to number of PCs which adequately summarizes the data set is usually just enough to explain some large percentages of the total variation of the variables [138] – values between 70 and 90% have been suggested, although smaller values are also acceptable if the number of variables of subjects increases. Another approach is to exclude PCs with variances less than one, but there are criticisms to this as well [138].

Before PCA can be done to a $(n \times p)$ data matrix (D) which contains n reflectance values at each p wavelength, the data matrix D must be centered so that it varies about zero [136]. This is done by replacing each value of reflectance by its difference to the average reflectance at a given wavelength. The resulting matrix X is thus represented by [109]:

$$X = TP' + E \quad (3.4)$$

where, T is the score matrix, P' is the transposed loading matrix, and E is the residual matrix. The loadings are the link between the wavelength coordinates of the X matrix and the PC space and the residual is the amount of spectral information that is not described by the PCs. Algorithms for PCA have been proposed by Wold [139] and Smith [140], and has been adapted below:

Step (1). Get the data, in this case, infrared spectra from the samples which contain n reflectance values at each p wavelength, and form a $(n \times p)$ data matrix (D).

Step (2). Subtract the mean reflectance ($\bar{n}$) from each value of the reflectance at a given wavelength, so that all the n values have $\bar{n}$ subtracted.

Step (3). Center the data matrix (D) by replacing each reflectance value with the resultant $(n - \bar{n})$ so that it varies about zero. This results in a matrix X represented by $X = TP' + E$.

Step (4). Calculate the loading vector p' (the rows in matrix P') as $p' = t'X/t't$ for the score vector t (the columns in matrix T) for each dimension. The i th element in p' can be interpreted as the slope in the linear regression of t on the i th row in X .

Step (5). Normalize p' to length one by dividing the vector by its length, i.e. multiplying by $c = 1/\sqrt{p'p}$.

Step (6). Calculate a new score vector $t = Xp/p'p$. The i th element in t can be interpreted as the slope in the linear regression of p' on the i th row in X .

Step (7). Check for convergence between all elements in two consecutive score vectors. If convergence occurs, continue with next step, otherwise return to step 4.

Step (8). Construct the residual matrix $E = X - tp'$, and use E as X in the next dimension.

Twenty random samples from each category of products were chosen for analysis, except for the ginsenosides, jiegeng, and the ginseng samples from Hong Kong, in which all the samples were used. For the differentiation of quality among American and Asian ginsengs, all the samples were taken into account for the analysis. 727 points from the fingerprint region (2000-600 cm^{-1}) of the MIR spectra, as well as 1927 points from the NIR spectra were used for PCA calculations. Obvious outliers were removed from each analysis, and the remaining spectra were recalculated and analyzed.

3.3.4. Pearson Correlation Coefficient

The Pearson correlation coefficient (r) is a measure of how well two variables X and Y can be described by a linear equation [141], and is defined as [142]:

$$r = \frac{n \sum xy - \sum x \sum y}{\sqrt{[n \sum x^2 - (\sum x)^2][n \sum y^2 - (\sum y)^2]}} \quad (3.5)$$

The values range from -1 to 1 which indicate perfect negative and positive correlations respectively [141]. Hence, a higher r value would indicate a greater degree of correlation.

A library of ginseng root samples was created in the Shimadzu IRsolution 1.10[®] program (Creon Lab Control AG, Shimadzu Corporation Pte. Ltd., Asia Pacific), and the MIR and NIR spectra of the commercial products – ginseng tea and capsules, were compared to their corresponding ginseng root powders using Pearson correlation

coefficient analysis based on spectral similarity. The Hit Quality Index (HQI) score, as defined by the software program, expresses the similarity of an unknown spectrum and a reference spectrum in numeric value which ranges from 0 to 1000. It was based on a modification of the above formula:

$$HQI = \frac{\sum_{i=1}^m (S_i - \bar{S})(r_i - \bar{r})}{\sum_{i=1}^m (S_i - \bar{S})^2 \sum_{i=1}^m (r_i - \bar{r})^2} \quad (3.6)$$

where, m refers to the number of data points, S_i and r_i refer to the spectra of the unknown (in this case of the commercial product) and reference (ginseng root sample) respectively, and $\bar{S}$ and $\bar{r}$ refer to their corresponding mean values. A closer similarity between the spectra of the commercial product to their corresponding root samples was reflected by a higher HQI score.

The average HQI score was calculated based on twenty samples for each commercial product sample group to compare the similarity of the product to its corresponding ginseng root sample.

3.4. Summary of Experimental Procedure for Ginseng Analysis

In accordance to the experimental procedure described in this section, an analytical sensing strategy based on IR spectral wavelengths was set up for ginseng, as shown in Figure 3.1. This strategy differs from conventional FTIR techniques in terms of data processing and analysis, in which the original and second derivative spectra from

both the mid- and near-infrared regions were used for qualitative analysis, as well as for principal component and Pearson correlation analyses. Table 3.1 also provides a summary of the number of samples used and the types of data analysis that were carried out for each compound in this study.

Figure 3.1. Analytical sensing strategy for ginseng based on IR spectral wavelengths.

Table 3.1. Matrix showing number of samples and types of data analysis.

Types of samples	Number of samples tested				Data analysis		
	Mid-IR (4000-400cm ⁻¹)		Near-IR (7800-4000cm ⁻¹)		Qualitative Analysis	Principal component analysis	Pearson correlation analysis
	General Spectra	Second derivative spectra	General Spectra	Second derivative spectra			
Ginsenosides							
Rb ₁	3	3	3	3	✓	✓	
Rc	3	3	3	3	✓	✓	
Re	3	3	3	3	✓	✓	
Rg ₁	3	3	3	3	✓	✓	
F ₁₁	3	3	3	3	✓	✓	
Ginseng Species							
American ginseng (<i>Panax quinquefolius</i>)	50	50	50	50	✓	✓	
Asian ginseng (<i>Panax ginseng</i>)	50	50	50	50	✓	✓	
Adulterants							
Jiegeng (<i>Platycodon grandiflorum</i>)	10	10	10	10	✓	✓	
Sawdust	50	50	50	50	✓	✓	
Ginsengs from Hong Kong							
Grade 2 (G2)	10	10	10	10	✓	✓	
Grade 5 (G5)	10	10	10	10	✓	✓	
Grade 10 (G10)	10	10	10	10	✓	✓	
Commercial products							
American ginseng capsules	20	20	20	20	✓	✓	✓
Korean ginseng capsules	20	20	20	20	✓	✓	✓
American ginseng tea	20	20	20	20	✓	✓	✓

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Comparison of Ginsenosides

The IR spectra of five ginsenoside standards were analyzed in this study – ginsenosides Rb₁, Rc, Re, Rg₁, and pseudoginsenoside F₁₁. Among these, pseudoginsenoside F₁₁ has been used to distinguish American ginseng from Asian ginseng since some studies have reported that it is present in American ginseng but not in Asian ginseng [30,50,52].

The original MIR spectra of ginsenosides Rb₁, Rc, Re and Rg₁ were all similar, hence Rb₁ was taken as a reference to compare with F₁₁ (Figure 4.1). The peaks at ~3333 and ~1072cm⁻¹ were due to the stretching of the alcohol hydroxyl (OH) groups, while that of ~1377cm⁻¹ was due to alcohol OH bending. The absorption bands at ~2943-2893cm⁻¹ were due to aliphatic C-H stretching. The intense band at ~2353cm⁻¹ was the carbonyl (C=O) stretch of carbon dioxide, which varied with measuring conditions. The alkene (C=C) and aromatic C-C absorption bands occurred at ~1676 and ~1587cm⁻¹ respectively. The peak at ~1026cm⁻¹ indicated the presence of an aryl-O-CH₃ stretch, while the peak at ~667cm⁻¹ was due to the OH wagging of water. This could be due to moisture accumulation during the preparation of the ginsenoside pellets.

The structure of pseudoginsenoside F₁₁ can be distinguished from the other ginsenosides from its lack of aliphatic double bonds (Figure 2.2). This is shown in Figure 4.1 by the lack of prominent peaks at the region ~1676cm⁻¹. The OH peak at ~3332cm⁻¹ was also shifted to ~3423cm⁻¹ in F₁₁. Furthermore, there were more peaks in the regions of 2970-2881cm⁻¹, 1454-1361cm⁻¹ and 1128-1026cm⁻¹ when compared to Rb₁.

Figure 4.2 shows the second derivative MIR spectra of the ginsenosides. As can be seen, the spectra of the ginsenosides looked quite similar, with the exception of pseudoginsenoside F_{11} . There were seven peaks which were common to all the five ginsenosides: $\sim 1800 \pm 5 \text{ cm}^{-1}$, $\sim 1310 \pm 5 \text{ cm}^{-1}$, $\sim 1085 \pm 5 \text{ cm}^{-1}$, $\sim 1025 \pm 5 \text{ cm}^{-1}$, $\sim 885 \pm 5 \text{ cm}^{-1}$, $\sim 735 \pm 5 \text{ cm}^{-1}$ and $\sim 675 \pm 5 \text{ cm}^{-1}$. These peaks could be used as distinguishing features for Rb_1 , Rc , Re , Rg_1 , F_{11} ; and possibly other ginsenosides as well. However, Rg_1 had fewer but more prominent peaks than Rb_1 , Rc and Re in the region of ~ 1830 - 1310 cm^{-1} . Rg_1 also exhibited a peak at $\sim 930 \text{ cm}^{-1}$ which was absent in the other three ginsenosides.

Figure 4.2. Second derivative MIR spectra of ginsenosides Rb_1 , Rc , Rg_1 , Re and pseudoginsenoside F_{11} .

The spectrum of pseudoginsenoside F_{11} was different from the other ginsenosides, particularly in the region of ~ 1160 - 740 cm^{-1} , in which there were not only more number of peaks, but more distinct peaks as well. In this region, the peaks at ~ 1050 , 955 , 843 and

807cm⁻¹ were observed in the F₁₁ spectrum, but not repeated in the spectra of the other ginsenosides. The other four peaks at ~1685, 1653, 1353 and 1230cm⁻¹ were also specific to F₁₁. However, the peak at ~1595cm⁻¹ was less prominent in the spectrum of F₁₁ as compared to the other ginsenosides. Thus these differences in the second derivative MIR spectra could be used to distinguish F₁₁ from the other ginsenosides.

As shown in Figure 4.3, the second derivative NIR spectrum of Rb₁ was similar to that of Re, as with Rg₁ and Rc. In general, Rb₁ had higher peaks than Re, particularly in the regions of ~7350-7050cm⁻¹ and ~5610-5190cm⁻¹. In addition, the absorption bands at ~6230, 5655 and 5160cm⁻¹ could distinguish Rb₁ from Re as well, since they were more prominent for Rb₁. The differences between Rg₁ and Rc were shown from the prominent peak at ~5790cm⁻¹ in Rg₁, and the peak shift from ~5025cm⁻¹ in Rc to ~4980cm⁻¹ in Rg₁. Furthermore, the absorption bands in the regions of 6750-6450cm⁻¹ and 6450-6150cm⁻¹ were more distinct and uniform in the spectra of Rg₁ and Rc respectively, and hence could also be used to distinguish Rc from Rg₁.

Rb₁ and Re can be differentiated from Rc and Rg₁ by their peak patterns and positions in two regions: 7350-7050cm⁻¹ and 5610-5190cm⁻¹. In the former region, Rc and Rg₁ exhibited two distinct peaks at ~7330 and 7240cm⁻¹, while the peaks for Re and Rb₁ were at ~7270 and ~7195cm⁻¹. In the latter region, three peaks were observed for Re and Rb₁ at ~5370, 5295 and 5220cm⁻¹, but only two distinct peaks at ~5535±5cm⁻¹ and 5295±5cm⁻¹ were observed for Rc and Rg₁.

Figure 4.3. Second derivative NIR spectra of ginsenosides (a) Re and Rb₁; (b) Rc and Rg₁; and (c) Re, F₁₁ and Rb₁.

The NIR spectrum of F₁₁ was similar to those of Rb₁ and Re (Figure 4.3c), but its pattern similarity was closer to that of Re, as shown by the absorption bands at ~5550-5190cm⁻¹. The presence of a peak at ~7710cm⁻¹ could be used to distinguish F₁₁ from these two ginsenosides since this was the only peak observed in the region of 7770-7650cm⁻¹, in contrast with the other two ginsenosides which had two peaks instead. The peak at ~7380cm⁻¹ was not as prominent in the F₁₁ spectrum as compared to the spectra of the other two ginsenosides, and the peak for Rb₁ at ~5160cm⁻¹ was also not observed for F₁₁. Lastly, the peak at ~6255cm⁻¹ could also distinguish F₁₁ from the other two ginsenosides.

PCA of the original MIR and NIR spectra could not differentiate among the various ginsenosides. However, the plots of second derivative spectra (Figure 4.4) showed that the ginsenosides could be grouped into clusters. Variance by the two components explained 77% and 89% of the MIR and NIR dataset variation respectively. However, the clusters shown by the mid-IR dataset was clearer than those of the near-IR dataset. This could probably be due to the poor resolution of the overlapping combination bands and overtones of the near-IR spectra, as compared to the fundamental frequencies in the mid-IR region of the molecule [108]. Nevertheless, PCA analysis confirms the difference in the structure of pseudoginsenoside F₁₁ with the other ginsenosides, as shown by the clusters, particularly in the MIR region. Hence, this substantiates the fact that F₁₁ can be used to distinguish American ginseng from Asian ginseng.

Figure 4.4. 2D plots of ginsenosides. The plots display the 1st two PCs of (a) second derivative mid-IR spectra, PC1=58% vs PC2=19%; and (b) second derivative near-IR spectra, PC1=76% vs PC2=13%.

4.2. Comparison of Ginsengs

4.2.1. Differentiating between American and Asian Ginsengs

The IR spectra of the same ginseng species exhibited similar features in the study samples. The aliphatic C-H stretch occurred in the general mid-IR spectra of both the American and Asian ginsengs at $\sim 2934\text{--}2928\text{cm}^{-1}$. This was consistent with a study by Li et al. [43] which stated that the peak at $\sim 2928\text{cm}^{-1}$ was due to the stretching vibration of CH_2 - groups. In a study by Schulten H.-R. and Soldati F., it was reported that the absorption band at 3380cm^{-1} was due to OH for ginsenosides Rg₁ and Re [143]. This was also consistent with the results of this study in which the same stretch bands occurred at ~ 3379 and 3393cm^{-1} for the American and Asian ginsengs respectively. However, this band was not specific to only ginsenosides, but could be due to the hydrogen bonds of water molecules in moisture during the analysis, as well as any other samples which contained other saponins with the OH functional group. The absorption bands at the region of around 2350cm^{-1} was due to the C=O stretch of CO_2 and could be affected by

environmental conditions, hence its intensity varied widely during the measurements of the various samples and could not be used to distinguish among the samples.

Several studies [14,55] have claimed that *P. quinquefolius* and *P. ginseng* are closely related. This posed a challenge in the distinguishing of the ginsengs from their IR-spectra. A closer look at the fingerprint regions ($2000\text{-}600\text{cm}^{-1}$) (Figure 4.5) showed that there were minor differences which could be used to differentiate the two ginsengs, mainly in the regions of $1560\text{-}1480\text{cm}^{-1}$ and $1200\text{-}900\text{cm}^{-1}$. There were more peaks in the latter region of the spectrum of Asian ginseng than American ginseng. American ginseng also exhibited clear amide peaks at ~ 1540 and 1523cm^{-1} which were due to NH bending and CN stretching, but not Asian ginseng.

The peak at $\sim 1735\text{cm}^{-1}$ is due to the carbonyl (C=O) groups in volatile oils and other compounds, while the peaks at ~ 1024 and $\sim 1049\text{cm}^{-1}$ represent the C-O in the alcohol hydroxyl groups [43]. The peaks which fall in the range of $1150\text{-}940\text{cm}^{-1}$ are due to C-C and C-O vibrations, which is consistent with Li et al.'s findings of C-O-C groups within the range of 1100 and 940cm^{-1} [43]. The absorption bands at $\sim 1618\text{-}1631\text{cm}^{-1}$ are due to the C=C stretch of ginsenosides Rg₁ and Re [143]. Sculten and Soldati [143] had further postulated that the OH and C=C absorption bands were also characteristic for other ginsenosides. The bands at ~ 1334 and 1411cm^{-1} are due to CH₃ deformations, and the bands around the region of $1247\text{-}1102\text{cm}^{-1}$ are due to the C-O stretching of fats. Ring stretching of carbohydrates occurred at $\sim 927\text{cm}^{-1}$, while aromatic CH bending was observed at $\sim 857\text{-}757\text{cm}^{-1}$. Lastly, from the original spectra, unsaturated carbon twisting and bending was also observed at $\sim 700\text{cm}^{-1}$.

Figure 4.5. Magnification of MIR spectra of (a) American ginseng, (b) Asian ginseng, and (c) both ginsengs in the $2000\text{-}600\text{cm}^{-1}$ region.

Saita et al. [85] also attributed absorption bands at 3420cm^{-1} to hydroxyl (OH) groups, 2252cm^{-1} to conjugated triple bonds ($\text{C}\equiv\text{C}-\text{C}\equiv\text{C}$) [85], and 2720 and 1706cm^{-1} to aldehyde (CHO) groups. However, these bands could not be clearly distinguished in the present spectra. These bands could have been masked in the present spectra since the results obtained by Saita et al. were of compounds that were extracted and purified, while raw powdered ginseng roots were being used in the present study.

The second derivative spectra of the fingerprint regions of both ginsengs (Figure 4.6) showed differences, particularly at $\sim 2000\text{-}1800\text{cm}^{-1}$, $1600\text{-}1300\text{cm}^{-1}$ and $1100\text{-}960\text{cm}^{-1}$. There were more distinct peaks in the American ginseng spectrum in the $2000\text{-}1800\text{cm}^{-1}$ region, in contrast to the Asian ginseng spectrum which showed a relatively flat line. More prominent and distinct peaks were also seen in the $1600\text{-}1300\text{cm}^{-1}$ region of the Asian ginseng spectrum as compared to that of American ginseng which showed more peaks of lower intensity. Furthermore, there were two peaks at ~ 1073 and 668cm^{-1} in the Asian ginseng spectrum that was not observed in the American ginseng spectrum.

In this study, the absorption bands for carbohydrates I and III occurred at $924\pm 1\text{cm}^{-1}$ and $764\pm 1\text{cm}^{-1}$ respectively. This was similar to another study [43] in which the peaks at $917\pm 13\text{cm}^{-1}$, $891\pm 7\text{cm}^{-1}$ and $766\pm 10\text{cm}^{-1}$ indicated the absorption bands of carbohydrates. However, the carbohydrate II band at $\sim 891\text{cm}^{-1}$ was not seen in the spectra of the present samples. The same study also postulated that the peaks at ~ 1052 , 1021 and 862cm^{-1} were indicative of starch being present in the sample, and a stronger peak implied lower starch content. Although the three starch peaks were observed for Asian ginseng at ~ 1049 , 1021 and 860cm^{-1} , only two peaks were present at ~ 1050 and 850cm^{-1} for American ginseng. Studies [8,144] have shown that the components of ginseng vary in

different geographical regions, seasonal changes and cultural practices. This could probably explain the variation in the starch absorption bands. Nevertheless, this study further confirmed the presence of starch in the ginseng root powders sold in Singapore. Calcium oxalate [43] was also present in our samples as indicated by the peak at $\sim 1322 \pm 1 \text{ cm}^{-1}$.

Figure 4.6. Second derivative MIR spectra of American and Asian ginsengs showing spectral differences.

The following absorption bands were assigned to the near-IR spectra of the ginsengs: the bands at $7400\text{--}7050 \text{ cm}^{-1}$ were due to CH combination bands, $5910\text{--}5600 \text{ cm}^{-1}$ to CH stretch overtones, and $5370\text{--}5130 \text{ cm}^{-1}$ to OH overtones and combination bands. The moisture peaks were in the $5370\text{--}5070 \text{ cm}^{-1}$ range, with its peak at $\sim 5160 \text{ cm}^{-1}$ for Asian ginseng, and $\sim 5177 \text{ cm}^{-1}$ for American ginseng. This was similar to the peaks described by Ren and Chen [110], who claimed that the absorption bands for the moisture content of ginseng was found to be between 5832 and 4990 cm^{-1} , and its peak was at ~ 5160 and

5155 cm^{-1} . Samples with high water content will affect the complexity and interference of the OH absorption bands due to their hydrogen bonding, and solute-solvent interactions produced by water will also affect the spectra [65]. Moisture content of ginseng roots is an important quality index as it relates to its preserving characteristics. Microbial and enzymatic activities are also affected by the high moisture content in ginseng, and this may lead to a decrease in both its medicinal and commercial values. Thus, studies have been done on several methods such as hot-air drying, freeze-drying and steaming in ginseng processing in the attempt to reduce its moisture content [110].

The second derivative near-IR spectra of American and Asian ginsengs (Figure 4.7) showed pattern differences, particularly at four regions. Distinctive peaks were seen in the American ginseng spectrum at $\sim 6450\text{-}5550\text{cm}^{-1}$ and $5045\text{-}4290\text{cm}^{-1}$, as compared to that of Asian ginseng. In addition, the most intense absorption bands occurred at the $7350\text{-}7050\text{cm}^{-1}$ and $5490\text{-}5190\text{cm}^{-1}$ for both ginsengs, with American ginseng having more number of distinct peaks, as well as stronger peaks. The positions of the bands were also different for each type of ginseng. In the region of $7370\text{-}7050\text{cm}^{-1}$, the absorption bands occurred at ~ 7260 , 7190 , 7125 and 7075cm^{-1} for American ginseng and ~ 7340 , 7232 , 7155 and 7106cm^{-1} for Asian ginseng. Similarly, there were four absorption bands at ~ 5480 , 5380 , 5297 and 5202cm^{-1} in the American ginseng spectrum, but only three absorption bands at ~ 5448 , 5340 and 5245cm^{-1} in the Asian ginseng spectrum at the $5490\text{-}5190\text{cm}^{-1}$ region. Thus these four regions are also characteristic regions which could potentially be used to distinguish between the two ginsengs.

Figure 4.7. Second derivative NIR spectra of American and Asian ginsengs.

The two-dimensional (2D) plots of the original mid-IR spectra could not be used to differentiate the ginsengs. However, the near-IR spectra showed that the ginsengs could be distinguished from each other. The American ginsengs seemed to be separated into two different clusters based on their general spectra (Figure 4.8a), but further analysis on their second derivative NIR spectra (Figure 4.8b) showed that they formed a big cluster which could be differentiated from Asian ginseng, with the first two PCs explaining approximately 73% of the data set variation.

This differentiation could also be seen from their second derivative mid-IR spectra (Figure 4.8c), which reflects the fundamental vibrations of the functional groups present in the components of ginseng: 76% of the total variance could be explained by the first two PCs, with the first and second PCs consisting of 56% and 20% of the total variance

respectively. American ginseng seemed to have a smaller intra-class variance in Figure 4.8c, but in contrast, Asian ginseng had a smaller class variance in Figure 4.8b. This could be due to the difference in spectral domains in which the analyses were carried out. The ginseng that is sold in medicinal halls in Singapore is not cultivated locally, but imported from other countries. Since different growing environments and cultivation conditions may also affect the amount of active constituents such as ginsenosides and polysaccharides in ginseng roots [9,37], and furthermore, the name “Asian ginseng” includes other species which are originated from Asian countries like *Panax japonicus* (Japanese ginseng), *Panax notoginseng* (Sanchi ginseng) and *Panax sinensis* J. Wien [33,145]; this could possibly explain the class variation in ginseng spectra as well. Thus, although American ginseng is closely related to Asian ginseng, there is still a good separation between them based on PCA of their IR spectra.

Figure 4.8. 2D plots of American and Asian ginsengs. The plots display the 1st two PCs of (a) general NIR spectra PC1=61% vs PC2=33%; (b) second derivative NIR spectra PC1=49% vs PC2=24%; and (c) second derivative MIR spectra PC1=56% vs PC2=20%.

4.2.2. Differentiating Among the Different Grades of Ginsengs

During PCA of the mid-IR second derivative plots, it was observed that there seemed to be a wide variation in the American and Asian ginseng samples purchased in Singapore. A deeper analysis of their plots revealed that the ginsengs were actually separated into different classes indicative of their grades. As shown in Figure 4.9, the American ginseng samples could be grouped into low (G1-G2), medium (G3-G5) and

high (G6) grades, and the Asian ginseng samples into low (G1-G4) and high (G5-G7) grades. The 2D plots could explain up to 73% and 85% of the total variance for the American and Asian ginseng samples.

A search done on the Pubmed database with the keywords ‘grade’, ‘grades’, ‘grading’, ‘grading system’, ‘quality grades’, and ‘quality grading systems’ with ‘ginseng’ and ‘ginsenosides’ returned only three results, out of which none compared traditional grading systems of authenticating ginseng with the more sophisticated and well-established analytical techniques. This is a novel finding since no or little studies have correlated the grading of ginseng quality via traditional morphological means with analytical methods before. However, since only the mid-IR spectra was analyzed for this purpose, further work could be done to relate the various ginseng grades via traditional means of authentication based on morphology of their roots with their near-IR fingerprints.

Figure 4.9. 2D plots distinguishing the various grades of (a) American ginseng, PC1=50% vs PC2=23%; and (b) Asian ginseng, PC1=62% vs PC2=23%.

4.3. Comparison of American and Asian Ginsengs with Adulterants

4.3.1. Distinguishing from Sawdust

Wood may seem to be a simple substance, but its chemical components are actually quite complex [146]. Wood comes from trees, and there are two biological classifications of trees: softwoods and hardwoods. However, they do not refer to the hardness or density of the wood material [146] – softwoods are also known as gymnosperms due the fact that these trees produce “naked seeds”, while hardwoods are called angiosperms and they are trees which produce seeds that are enclosed in a nut or fruit. Softwoods are usually used for most commercial wood products, especially for structural applications [146].

The complexity of wood comes from its chemically heterogeneous nature. Its constituents can be divided into two categories known as the structural components which are the major components of wood, and non-structural components of low molecular mass [146,147]. The structural components include constituents such as cellulose, hemicellulose and lignin, while the non-structural components consist of the extractives and inorganic compounds [147]. About 50% of the dry weight of wood is made up of cellulose, which is composed of polymers of glucose molecules [146]. The bonds between these glucose molecules are very strong hence giving cellulose, and in turn wood, its strength. Hemicellulose, the second most abundant chemical component, accounts for approximately 15 to 25% of wood’s dry weight [146]. It is different from cellulose in that their polymers form branched chains rather than straight chains, and it is comprised of glucose and other water soluble sugars produced during photosynthesis, unlike cellulose which is made up of only glucose molecules. Lignin is the third most abundant chemical

component of wood and it constitutes about 15 to 30% of the dry weight of wood [146]. It is a three-dimensional polymer whose exact structure is not fully understood, but it occurs throughout the cell wall and is concentrated on the outside of cells and between cells, and it gives wood its property of stiffness. Extractives, on the other hand, are basically the various chemicals that are found in the cell walls and lumens of wood that are not its structural components [146]. They confer wood its properties of color, odor, taste, density, hygroscopicity, decay resistance and even flammability, and account for about 2 to 15% of the dry weight of wood. The extractives are called as such because they can be removed from wood by heating it in water or other organic solvents. Examples of extractives include tannins, oils, fats, starch, resins, gums and waxes.

Sawdust is defined by the Wikipedia encyclopedia [148] as a material that is composed of fine particles of wood produced from cutting with a saw. Sawdust was chosen for analysis in the current study as it looks similar to ginseng powder, and it is also an adulterant of ginseng dietary supplements [58]. A visual inspection by an untrained eye will not be able to distinguish sawdust from ginseng powder. This is especially important since both of these substances exhibit totally different properties, and will produce different physiological effects when taken by a person. Hence, part of the study aim was to be able to distinguish this “morphological fake” from the real ginseng herb.

The region of the MIR spectra above 2000cm^{-1} could not be used for confirmatory distinguishing of ginseng and sawdust. This was because the carbon dioxide, alkyl CH and hydrogen bonded hydroxyl (OH) absorption bands at $\sim 2919\text{cm}^{-1}$, $\sim 2344\text{cm}^{-1}$ and $\sim 3428\text{cm}^{-1}$ respectively were not sharp and their positions were also very near those of the

ginseng spectra. This could cause misinterpretation of the bands if the sample was an unknown.

A magnification of the region between 2000-600 cm^{-1} (Figure 4.10) showed that the peaks at ~ 1745 and $\sim 1508\text{cm}^{-1}$ were indicative of the C=O and aromatic C=C functional groups respectively [147]. The complex fingerprint region below 1460 cm^{-1} was postulated to be contributed from the various vibration modes in carbohydrates and lignin, except for the band near 1510 cm^{-1} , which was relatively pure [147]. Other C-H vibration modes were also observed in the regions 1560-1360 cm^{-1} and 900-740 cm^{-1} , while the prominent bands at 1320-1000 cm^{-1} and below 665 cm^{-1} were caused by C-O and C=C vibrations.

Figure 4.10. General MIR spectra of sawdust in the range of 2000-600 cm^{-1} .

The fingerprint region ($1800\text{-}900\text{cm}^{-1}$) which was identified by Freer et al. [147] to be distinctive to wood was also observed in the present spectra in Figure 4.11. The sawdust spectrum in this region was observed to be very different from both Asian and American ginsengs. There were many more absorption bands in the sawdust spectrum, and these bands were more prominent and distinctive as compared to the ginsengs' spectra. This fingerprint region that could distinguish the three samples was extended to 760cm^{-1} in their second derivative spectra. Although the whole sawdust spectrum was visibly different from the ginsengs, there are a few areas worth highlighting. There were ten peaks which were not only prominent, but unique to sawdust at ~ 1752 , 1720 , 1595 , 1556 , 1543 , 1509 , 1270 , 955 , 895 and 807cm^{-1} . Furthermore, in the region of $1100\text{-}1000\text{cm}^{-1}$, the sawdust spectrum exhibited two peaks (~ 1062 and $\sim 1027\text{cm}^{-1}$) in contrast to the Asian ginseng spectrum which exhibited three peaks (~ 1073 , 1049 and 1021cm^{-1}), and the American ginseng spectrum which exhibited only one peak ($\sim 1050\text{cm}^{-1}$).

The NIR spectra of American ginseng, Asian ginseng and sawdust are shown in Figure 4.12. As with the American and Asian ginseng spectra, the shapes and slopes of sawdust's general spectrum were also erratic with a wide variation. Hence it was not used for visual distinguishing of ginsengs and their adulterants. A look at their second derivative spectra showed that the peak positions in the region of $7350\text{-}7050\text{cm}^{-1}$ were different among the three samples and four of the peaks at ~ 6650 , 6570 , 6499 and 6435cm^{-1} were more prominent in sawdust. Their spectral fingerprints below 6435cm^{-1} were also different, except for the four peaks at $\sim 5480\pm 1$, 5375 ± 5 , 5296 ± 1 and $5202\pm 1\text{cm}^{-1}$, in which both sawdust and America ginseng were similar.

Figure 4.11. Comparison of (a) general and (b) second derivative MIR spectra of sawdust with American and Asian ginsengs.

Figure 4.12. Comparison of the second derivative NIR spectra of sawdust with American and Asian ginsengs.

PCA analysis was not able to separate the sawdust and ginsengs into classes based on their general NIR spectra. However, the cluster of sawdust samples was clearly shown from the MIR spectra (Figure 4.13). A further differentiation among the American and Asian ginseng samples was shown in the second derivative 2D plots of both the MIR and NIR spectra. However, the boundaries of the classes in the NIR plot were not as distinct. The first two PCs represent 85% and 74% of the total variance in the MIR and NIR datasets respectively. The American and Asian ginseng samples could be seen to form closer clusters with each other than with the sawdust samples. Thus, the differences in chemical composition between sawdust and ginseng could be easily identified from their IR spectra. It was noted that further analysis could be carried out based a wider range of

NIR wavelengths as this would probably be able to separate the samples into more distinct classes.

Figure 4.13. 2D plots of sawdust, American and Asian ginsengs. (a) General MIR spectra, PC1=82% vs PC2=10%; (b) Second derivative MIR spectra, PC1=74% vs PC2=11%; (c) Second derivative NIR spectra, PC1=51% vs PC2=23%.

4.3.2. Distinguishing from *Platycodon grandiflorum* (Jiegeng)

Jiegeng or *Platycodon grandiflorum* A. De Candolle belongs to the family of Campanulaceae [149,150], and is also called Kikyuu in Japanese [150]. It is also known as a balloon flower [151] that is widely spread in East Asia including China, Japan and Korea [152]. Its roots are commonly used in traditional Chinese medicine for its

antitussive and expectorant effects [149,150,152,153]. It is also used for bronchitis, pharyngolaryngitis, asthma, pulmonary tuberculosis, diabetes, hyperlipidemia, and other inflammatory diseases [149,151,153]. Thus, it is categorized in TCM practice as a herb which has expectorant, antitussive and lung ventilation effects (化痰止咳平喘药) [154-156]. The main chemical constituents of this herb are also the triterpenoid saponins, out of which platycodins A, C and D account for most of the saponins [150]. The other saponins in jiegeng include the platycosides, deapioplatycodins, and polygalacins, among others [150,151]. All these saponins bear a similar four-ringed carbon skeleton as the ginsenosides which are the saponins of ginseng.

Due to the similarity of the active ingredients of jiegeng and ginseng, it has been used as a cheaper herbal substitute for ginseng [157]. As this herb is also widely available in Singapore, it was chosen in the present study to assess the feasibility of distinguishing this similar adulterant from ginseng.

The same absorption bands that were seen in the ginseng spectra were also observed in jiegeng in the mid-IR region above 2000cm^{-1} . The jiegeng samples also presented a peak at $\sim 3391\text{cm}^{-1}$ which was postulated by Schulten et al. [143] to be due to ginsenosides. However, their results were based on pure fractions of ginsenosides obtained from ginseng, and they did not compare the peaks with other adulterants. This particular peak can also be due to the platycodins in jiegeng, since that makes up the majority of its saponins. As with the wood samples, this peak alone was not able to distinguish between ginseng and jiegeng.

A magnification of the fingerprint region from $2000\text{-}600\text{cm}^{-1}$ (Figure 4.14) showed a high similarity between jiegeng and the two ginsengs. The main difference was

the two equal intensity absorption bands at ~ 1054 and 1031cm^{-1} and the three prominent bands at ~ 859 , 817 and 777cm^{-1} .

Figure 4.14. Comparison of the magnified fingerprint region ($2000\text{--}600\text{cm}^{-1}$) of American and Asian ginsengs with jiegeng.

From their second derivative spectra (Figure 4.15), it was observed that the jiegeng spectrum was more similar to that of Asian ginseng than American ginseng. The region between 1600 and 1360cm^{-1} was able to differentiate jiegeng from American ginseng due to the pattern differences in their spectra, but it was very similar to Asian ginseng. The $\sim 1446\text{cm}^{-1}$ peak observed for American ginseng was not present in the Asian ginseng and jiegeng spectra. Jiegeng could also be distinguished from both ginsengs by a prominent peak at $\sim 820\text{cm}^{-1}$ and an absence of peak at $\sim 1205\pm 1\text{cm}^{-1}$. Furthermore, the spectral region between 1100 and 1000cm^{-1} was unique to the ginsengs, with jiegeng exhibiting two peaks (~ 1064 and 1025cm^{-1}) that were almost similar to those of sawdust

(Figure 4.11b). The NIR second derivative of jiegeng looked even more similar to Asian ginseng from the strong absorption bands at $\sim 7350\text{-}7050\text{cm}^{-1}$ and $5550\text{-}5130\text{cm}^{-1}$. However, the small absorption bands at ~ 7530 , 7450 and 4140cm^{-1} were able to distinguish it from the ginsengs, whose peaks were at $\sim 7495 \pm 5\text{cm}^{-1}$. The American ginseng spectrum also had a peak at $\sim 7675\text{cm}^{-1}$ which was not seen in the other two spectra.

The 2D score plots (Figure 4.16) of the MIR spectra were able to show separate clusters of jiegeng, American and Asian ginsengs. The second derivative plot was able to show a more distinct cluster of jiegeng with 80% of the total variance being explained. The general NIR spectra could not fully separate jiegeng from Asian ginseng. Its second derivative NIR plot, however, showed that even though the Asian ginseng samples seemed to be surrounding the jiegeng samples, they could still be separated into clear clusters, hence showing the difference in their species. Further testing could be done on more jiegeng samples and on a wider range of near-IR wavelengths to confirm this finding.

Figure 4.15. Comparison of second derivative (a) MIR and (b) NIR spectra of American and Asian ginsengs with jiegeng.

Figure 4.16. 2D plots of jiegeng, American and Asian ginsengs. (a) General MIR spectra, PC1=76% vs PC2=14%; (b) Second derivative MIR spectra, PC1=51% vs PC2=29%; and (c) Second derivative NIR spectra, PC1=48% vs PC2=23%.

Overall, the 2D plot of the second derivative MIR spectra was able to clearly show the classes of jiegeng, American and Asian ginsengs. This could be explained by the different functional group moieties of the constituents in jiegeng, Asian and American ginsengs such as their triterpenoid saponins. Differentiation of the samples based on their NIR spectra was not as good as their MIR spectra. Further tests could be carried out on more samples of jiegeng, as well as on a wider NIR wavelength range to confirm the usefulness of IR wavelengths in differentiating jiegeng from the ginsengs.

4.4. Further Establishment of the IR-based Sensing Technique

The current “gold practice standard” for ginseng analysis is the HPLC-MS detection of ginsenosides. A flowchart illustrating this method of analysis for ginseng is shown in Figure 4.17. As can be seen, this method of analysis is tedious and time consuming due to the preparation of ginseng samples. Several studies have demonstrated that the extraction process can range between 1h and 20h for ultrasonic and soxhlet extractions respectively [10,17,52,81], thus it is not suitable for analyzing large quantities of ginseng samples. Furthermore, HPLC analysis would have to be carried out under gradient flow conditions which takes approximately 1h [5,11,15,37]. In addition, preparation of stock solutions of ginsenoside standards are also required as references for comparison [11,15,37,52], and the presence of malonyl-ginsenosides has to be taken into account for analysis. This is because the malonyl-ginsenosides tend to undergo demalonylation by heat and hydrolysis, and this would affect the quality of the ginseng samples. Thus, care has to be taken to convert them to their respective neutral ginsenosides so that they can be more readily determined by the HPLC analysis [5,8,81].

Figure 4.17. Flowchart illustrating the current practice of HPLC-MS analysis of ginseng.

The IR-based sensing strategy presented in this study, as shown previously in Figure 3.1, was not only able to successfully distinguish between American and Asian ginsengs, but also differentiate them from two adulterants – sawdust and *Platycodon grandiflorum* (jiegeng). Unlike its HPLC-MS counterpart, the main advantages of this technique are its rapidness, ease of use and the lack of need for sample preparation. In this study, extraction of the ginseng samples was not required. The ginseng samples could immediately be mixed with KBr powder and pressed into pellets after being ground into powder. The time taken for the preparation of each ginseng sample was approximately 10mins, while the detection process by the IR spectrometer was approximately 2mins. This meant that the sensing strategy proposed in this study was suitable for analyzing large quantities of ginseng samples, as previously illustrated in Table 3.1.

In contrast to the HPLC-MS methodology in which the malonyl-ginsenosides had to be taken into account, this was not of a major cause of concern in the present study. Although ginsenosides are considered the major active ingredients in ginseng, it is known that the pharmacological activity of ginseng is due to the mixture of its constituents, and not a single compound [15,37]. The sensing strategy proposed in this study took the whole ginseng sample, rather than just the ginsenosides, into account during the analysis process.

For further establishment of the IR-based sensing technique used in this study, more tests were carried out on three grades of ginsengs from Hong Kong and on three commercial products. The purpose of these tests was to confirm if the proposed technique could be used to identify unknown samples of ginseng and discriminate commercial ginseng products. The identities of the types of ginsengs were not made known during the

time of purchase, but the test results were subsequently verified by Mr. Gary Pang, district manager of Hockhua Ginseng Birdnest Trading Enterprises [32].

4.4.1. Comparison of the Three Grades of Ginsengs

Three grades of ginsengs were purchased from a shop in Hong Kong and analyzed according to the proposed sensing technique. They were identified by the shopkeeper as being of grades 2 (G2), 5 (G5) and 10 (G10) in increasing order of quality, but their identities were not revealed at the time of purchase. Figure 4.18 shows the mid-IR spectra of the three grades of ginsengs. The region above 2000cm^{-1} was similar to the spectra seen before in the ginsengs obtained from Singapore with the same absorption bands occurring at $\sim 2356 \pm 20\text{cm}^{-1}$ for carbon dioxide, $\sim 2927 \pm 1\text{cm}^{-1}$ for alkyl CH and $\sim 3403 \pm 3\text{cm}^{-1}$ for alcohol OH groups. The range of peak positions and intensities were the largest for the carbon dioxide absorption bands due to the variation in the room condition in which the analysis was carried out. Hence the region above 2000cm^{-1} was not useful for the identification of the samples.

Interestingly, from Figure 4.18a, the spectrum of the medium grade G5 ginseng seemed to look different as compared to the other two spectra. However, a closer look at the fingerprint region of $2000\text{-}600\text{cm}^{-1}$ revealed that its spectrum was more similar to that of the G2 than the G10 ginsengs. There were five peaks in the region of $1200\text{-}980\text{cm}^{-1}$ and their positions were almost similar to those of the G2 ginseng, with absorption bands at $\sim 1151 \pm 2$, 1104 ± 1 , 1076 ± 2 , 1046 ± 1 and $1019 \pm 3\text{cm}^{-1}$. The higher grade G10 ginseng had only four peaks at ~ 1141 , 1105 , 1047 and 1002cm^{-1} . The amide peaks which were present in the $1550\text{-}1520\text{cm}^{-1}$ region of the G10 ginseng were also not clearly distinguishable in

the G5 ginseng. This region of the G5 ginseng looked more similar to the G2 ginseng with its absence of amide peaks.

The second derivative spectra in the fingerprint region (Figure 4.18b) were also different. There were six peaks in the spectrum of the G2 ginseng at ~ 1155 , 1108, 1078, 1047, 1020 and 990cm^{-1} , but only four peaks at ~ 1155 , 1110, 1050 and 992cm^{-1} in the G10 ginseng spectrum. This region of the G5 ginseng spectrum was also more similar to that of the G2 ginseng with six peaks at ~ 1160 , 1112, 1084, 1045, 1015 and 994cm^{-1} , even though the peak at $\sim 1015\text{cm}^{-1}$ was not as prominent. The 1084cm^{-1} peak observed in the G5 ginseng spectrum was not clearly distinguishable in the G10 ginseng spectrum. However, a similar peak at $\sim 1078\text{cm}^{-1}$ occurred in the G2 ginseng spectrum instead. In the $1700\text{-}1500\text{cm}^{-1}$ region, the peaks at ~ 1600 , 1547, 1528 and 1518cm^{-1} were more prominent in the G10 ginseng spectrum as compared to the others. Furthermore, the spectral pattern of the G5 ginseng in this region was more similar to the G2 ginseng than the G10 ginseng.

The general spectra of the ginsengs in the near-IR region were not useful in their identification as they appeared to be erratic. However, a differentiation to the second derivative revealed some interesting information, as shown in Figure 4.19. The two regions of $7350\text{-}7050\text{cm}^{-1}$ and $5550\text{-}5190\text{cm}^{-1}$ revealed contrasting information about the identity of the medium grade G5 ginseng. The absorption bands at the former region were similar to those of G2 ginseng, but they were more similar to the higher grade G10 ginseng at the latter region. During analysis of the former region, it was observed that the four peaks of the G5 ginseng at $\sim 7338 \pm 2\text{cm}^{-1}$, $7230 \pm 1\text{cm}^{-1}$, $7149 \pm 1\text{cm}^{-1}$ and $7099 \pm 1\text{cm}^{-1}$ were almost similar to the G2 ginseng in terms of peak positions and intensities. However,

the absorption bands of the G5 ginseng at $\sim 5382 \pm 6 \text{ cm}^{-1}$, $5286 \pm 4 \text{ cm}^{-1}$ and $5193 \pm 3 \text{ cm}^{-1}$ were more similar to the G10 ginseng in the latter region ($5550\text{--}5190 \text{ cm}^{-1}$).

Figure 4.18. MIR spectra of the G2, G5 and G10 ginsengs. (a) General spectra; and (b) Second derivative spectra of the fingerprint region ($2000\text{--}600 \text{ cm}^{-1}$).

Figure 4.19. Second derivative NIR spectra of the G2, G5 and G10 ginsengs.

Although the identity of the G5 ginseng could not be concluded based on NIR spectral similarity, PCA of the spectra of the three grades of ginsengs revealed that they could all be separated into distinct clusters, as shown in Figure 4.20. The three grades of ginseng could be distinguished from their near-IR spectra with their plots explaining more than 90% of the dataset. However, PCA of the MIR fingerprint region of the ginseng spectra showed that the G5 ginseng was of closer resemblance to the G2 ginseng than the G10 ginseng. The 2D plot of their general MIR spectra did not manage to show a clear separation between the G2 and G5 ginseng samples, and the plot of their second derivative MIR spectra showed that the G2 ginseng samples separated the other clusters of ginsengs. This was an interesting phenomenon which warranted further analysis of the ginseng samples.

Figure 4.20. 2D plots of the G2, G5 and G10 ginsengs. The plots display the 1st two PCs of (a) general MIR spectra, PC1=73% vs PC2=22%; (b) second derivative MIR spectra, PC1=60% vs PC2=23%; (c) general NIR spectra, PC1=80% vs PC2=15%; and (d) second derivative NIR spectra, PC1=78% vs PC2=16%.

4.4.2. Identification of the Three Grades of Ginsengs

The IR spectra of the three grades of ginsengs were compared with those of the American and Asian ginsengs purchased from Singapore, as previously described in section 4.2.1. From their fingerprint mid-IR regions, the G2 and G10 ginsengs looked similar to the Asian and American ginsengs respectively. However, the absorption bands were more intense in G2 and G10 varieties, as shown in the boxed regions in Figure 4.21. The amide absorption bands in the region of $1550\text{--}1520\text{cm}^{-1}$ were more prominent in the

G10 ginseng, as were the C-C and C-O vibrations in the $1100\text{-}950\text{cm}^{-1}$ region. Similarly, the C-C and C-O absorption bands in the region of $1220\text{-}1000\text{cm}^{-1}$ were more obvious in the G2 ginseng samples.

Figure 4.21. Comparison of general MIR spectra of the G10 and G2 ginsengs with American and Asian ginsengs. (a) G10 and American ginsengs; and (b) G2 and Asian ginsengs.

In general, it was observed from the second derivative spectra that the G2 and G10 ginsengs' spectra looked alike to the spectra of the Asian and American ginsengs respectively, except for a few minor differences. The second derivative mid-IR spectra of the ginsengs are shown in Figure 4.22. The G10 ginseng had an additional peak at $\sim 1600\text{cm}^{-1}$ which was not clearly distinguishable in the spectrum of the American ginseng. The peak at $\sim 1446\text{cm}^{-1}$ was also absent from the G10 ginseng. In addition, the American ginseng exhibited a broad band at $\sim 1378\text{cm}^{-1}$ in contrast to the sharp peak at $\sim 1372\text{cm}^{-1}$ for the G10 ginseng. The spectra of the G2 and Asian ginsengs were almost similar, except for the absorption band at $\sim 1075 \pm 3\text{cm}^{-1}$, which was more prominent in the spectrum of the G2 ginseng.

From their second derivative NIR spectra (Figure 4.23), the intensity of bands for the American ginseng was higher than its G10 counterpart, while this was the opposite for the Asian and G2 ginsengs. As shown in part (b), the spectra of the G2 and Asian ginsengs were highly similar in their peak positions. However, there were minor distinguishing factors for the G10 and American ginsengs (part a) – the five bands at ~ 7125 , 7075 , 5480 , 5202 and 5170cm^{-1} were more obvious in the American ginseng spectrum than the G10 ginseng spectrum, which showed only two obvious peaks at ~ 7065 and 5196cm^{-1} .

Figure 4.22. Comparison of second derivative MIR spectra of the G10 and G2 ginsengs with American and Asian ginsengs. (a) G10 and American ginsengs; and (b) G2 and Asian ginsengs.

Figure 4.23. Comparison of second derivative NIR spectra of the G10 and G2 ginsengs with American and Asian ginsengs. (a) G10 and American ginsengs; and (b) G2 and Asian ginsengs.

Further PCA analysis was carried out on the ginseng spectra, and their 2D plots are shown in Figure 4.24. The American and G10 ginsengs could not be classified based on their general MIR spectra. However, there was a distinction from their NIR spectra, in which the plot (part **a**) explained up to 95% of the total variance. The American ginseng samples varied more widely, while those from the G10 ginseng seemed to form a tight cluster. A closer look at their second derivative plots (parts **b** and **c**) showed that this cluster was within the larger cluster of American ginsengs. The NIR plot was able to show this cluster with the first two principal components explaining 71% of the total variation, but this could only be seen from the first three PCs of the MIR plot, from which approximately 75% of the dataset could be explained, including 34% by the first PC. This showed that despite minor differences between the American and G10 ginseng samples, they could still be identified as being of the same species, and that the G10 ginseng samples belonged to the *P. quinquefolius* category. This result was subsequently verified by Mr. Gary Pang, district manager of Hockhua Ginseng Birdnest Trading Enterprises, who confirmed that the G10 ginseng was American ginseng [32]. The wide variation among the ginsengs obtained from Singapore was probably due to the fact that they were of different grades, and this reflected a difference in the chemical composition of their roots which could have been affected by different growing environments. Further testing could be done in future studies using more sophisticated techniques such as HPLC-MS, genomic or proteomic methods to confirm the identity of the G10 ginseng.

Figure 4.24. 2D plots of the G10 and G2 ginsengs with American and Asian ginsengs. (a) General NIR spectra, PC1=65% vs PC2=30%; (b) Second derivative NIR spectra, PC1=44% vs PC2=27%; and (c) Second derivative MIR spectra, PC1=34% vs PC2=27% vs PC3=14% of the G10 ginsengs; (d) Second derivative MIR spectra, PC1=44% vs PC2=29%; and (e) Second derivative NIR spectra, PC1=53% vs PC2=26% of the G2 ginsengs.

Unlike the plots for the American ginsengs, the Asian and G2 ginsengs could not be classified easily based on PCA of their general spectra. This could imply that there was a higher similarity between them. However, the minor differences from their second derivative spectra were more obvious in their 2D plots (parts **d** and **e**) which were able to distinguish the cluster of G2 ginseng samples. The total explained variance of the second derivative MIR and NIR plots were 73% and 79% respectively. As with the G10 ginsengs, the clusters of G2 ginsengs were also more compact, and fell within the bigger Asian ginseng cluster. Thus, the G2 ginseng was identified as belonging to the *P. ginseng* category. This result was also subsequently confirmed by Mr. Gary Pang, who identified the G2 ginseng as Asian ginseng. Similarly, more tests could be carried out on the G2 ginseng with sophisticated testing methods to further confirm its identity.

It was mentioned earlier in section **4.4.1** that even though the MIR spectrum of the G5 ginseng looked similar to that of the G2 ginseng, its NIR spectrum could not clearly establish its identity since the absorption bands at the 7350-7050 cm^{-1} region looked similar to those of the G2 ginseng, but it was more similar to the G10 ginseng at the 5550-5190 cm^{-1} region. Thus, a comparison of these two regions in its second derivative NIR spectrum was also made with the Asian and American ginsengs purchased from Singapore. The results are shown in Figure **4.25a**. In the 5550-5130 cm^{-1} region, the peak positions at $\sim 5379 \pm 1$, 5291 ± 6 and $5196 \pm 7 \text{cm}^{-1}$ were similar to those of American ginseng, but the bands in the 7350-6870 cm^{-1} region were more alike to those of Asian ginseng. The exception was the peak at $\sim 7080 \pm 5 \text{cm}^{-1}$ which was nearer to the American ginseng peak. PCA of the spectra (Figures **4.26a** and **b**) showed that the G5 ginseng samples could be

separated from both the American and Asian ginseng classes within the first two PCs, which accounted for 73% and 72% of the dataset variations.

The MIR fingerprint regions of the American, Asian and G5 ginsengs were also compared since the difference in chemical composition of the ginsengs would be reflected in their fundamental vibrations of their mid-IR spectra. Hence, this region would be more important in determining the classification of the G5 ginseng based on its chemical properties. From Figure 4.25b, it can be seen that there were four regions that enabled the comparison of the G5 ginseng spectrum with those of the Asian and American ginsengs. The absorption bands in the regions of 1800-1700 and 1600-1500 cm^{-1} , which represented the carbonyl and amide groups respectively, were more obvious in the American ginseng spectrum. Comparison of these regions showed that the G5 ginseng spectrum looked more similar to the Asian ginseng spectrum. The absorption peaks in the region which represented CH_3 deformations at 1480-1320 cm^{-1} were also more similar to that of Asian ginseng. The most distinguishable area of the spectra was probably between 1180 and 960 cm^{-1} , which was due to the C-C and C-O vibrations of fats, alcohols and aromatic ethers. The spectra of the Asian and G5 ginsengs in this region were not only very similar, but their peaks were also very prominent as compared to those for American ginseng.

The overall spectral fingerprint of the American ginseng's second derivative spectrum (Figure 4.25c) was the most dissimilar among all the three ginsengs. The three absorption peaks in the American ginseng spectrum (~ 1546 , 1528 and 1512cm^{-1}) appeared as one broad peak at $\sim 1515 \pm 2\text{cm}^{-1}$ in the other two ginseng spectra. In the region of 1500-1400 cm^{-1} , there were two prominent peaks at $\sim 1454 \pm 3\text{cm}^{-1}$ and $\sim 1409 \pm 1\text{cm}^{-1}$ in the spectra of the Asian and G5 ginsengs, but the four peaks at ~ 1477 , 1464 , 1446 and

1410 cm^{-1} in the American ginseng spectrum were less prominent. Furthermore, the two peaks at $\sim 1367 \pm 2 \text{cm}^{-1}$ and $\sim 1239 \pm 2 \text{cm}^{-1}$ were sharper in the Asian and G5 ginseng spectra, while the equivalent peaks in the American ginseng spectrum (~ 1378 and 1242cm^{-1}) were broader. Lastly, in the region of 1100-1000 cm^{-1} , there was only one peak ($\sim 1050 \text{cm}^{-1}$) for American ginseng, but three peaks ($\sim 1079 \pm 6 \text{cm}^{-1}$, $\sim 1047 \pm 2 \text{cm}^{-1}$ and $\sim 1018 \pm 3 \text{cm}^{-1}$) for the Asian and G5 ginsengs. Hence, it could be deduced that the G5 ginseng was closer to the *Panax ginseng* rather than the *Panax quinquefolius* species.

The 2D score plots of their mid-IR fingerprints (Figures 4.26c and d) also revealed interesting information. Although PCA of the general spectra could not separate the ginsengs into classes, their second derivative spectra showed clear clusters with 81% and 76% of the total variability being explained by the first two PCs. It could be seen that the separation of the G5 ginseng class was more distinct from the American ginseng class than the Asian ginseng class. Furthermore, the Asian ginseng cluster was a lot larger, almost encompassing the G5 ginseng cluster. This could imply the wide variation in the chemical composition of Asian ginsengs sold in Singapore, possibly due to the different growing environments of the ginsengs before they were imported into this country.

Thus, based on the spectral fingerprints and PCA score plots, the G5 ginseng was also identified as *Panax ginseng*, and its identity verified by Mr. Gary Pang. However, with more well-established and sophisticated testing methods which are currently available, the identity of the G5 ginseng could be further confirmed with HPLC-MS, genomic or proteomic methods in future studies.

Figure 4.25. Comparison of (a) second derivative NIR spectra; (b) general MIR spectra; and (c) second derivative MIR spectra of the G5 ginseng with American and Asian ginsengs.

Figure 4.26. 2D plots comparing the G5 ginseng with American and Asian ginsengs. (a) Second derivative NIR spectra of G5 and American ginsengs, PC1=49% vs PC2=24%; (b) Second derivative NIR spectra of G5 and Asian ginsengs, PC1=48% vs PC2=24%; (c) Second derivative MIR spectra of G5 and American ginsengs, PC1=72% vs PC2=9%; and (d) Second derivative MIR spectra of G5 and Asian ginsengs, PC1=47% vs PC2=29%.

4.4.3. Comparison of Commercial Ginseng Products with Ginseng Root Samples

Further analysis was also carried out on three ginseng products to show that the IR-based technique was not only capable of analyzing pure ginseng roots, but on commercial ginseng products as well.

Two types of ginseng capsules and one type of ginseng tea were used in this study. The two types of capsules were of the same brand, and their labels claimed that they

contained pure American white ginseng and Korean ginseng powders. Korean ginseng belongs to the category of Asian ginseng, while American white ginseng belongs to the American ginseng category. Their second derivative spectra are shown in Figure 4.27. From their spectra, the capsules can be clearly differentiated from each other. The American white ginseng capsule seemed to have more absorption bands in the MIR region, but the bands for the Korean ginseng capsule were better separated. In contrast, the absorption bands in the NIR region seemed to be more prominent and better differentiated in the American white ginseng capsule spectrum, especially at $\sim 5550\text{-}5250\text{cm}^{-1}$, which was one of the distinguishing regions for both types of capsules.

PCA was then carried out on their MIR second derivatives to see if the capsules themselves could be clustered separately from each other, since the MIR region contained more information about the functional groups of the constituents in the capsules. This is shown in Figure 4.28. From the plot, the Korean ginseng capsule cluster could be differentiated from the American white ginseng capsule cluster within the first three principal components, with 79% of the total variance being explained, including up to 49% for the first PC. Thus, the powder in the capsules could be differentiated from their IR spectra even though they were identical in appearance.

Figure 4.27. Comparison of the second derivative spectra of the (a) MIR fingerprint region and (b) NIR region of American white ginseng and Korean ginseng capsules.

Figure 4.28. 2D plot of the American white ginseng and Korean ginseng capsules, PC1=49% vs PC2=20% vs PC3=10%.

The second derivative IR spectra of the capsules were also compared with those of American and Asian ginseng roots. Their Pearson correlation coefficients (r), reflected as Hit Quality Index (HQI) scores in Tables 4.1 and 4.2, were calculated based on spectral pattern similarity between the capsules and the root samples. As the products would have undergone various processing conditions, as well as the addition of other excipients, their IR spectra would be expected to be different from the ginseng roots. Therefore, the objective of carrying out a Pearson correlation analysis was to deduce the degree of similarity between the spectra of the products and ginseng roots.

It was observed that for both the capsules, their general spectra had higher correlation scores than their second derivatives. These were expected results due to the fact that there are many overlapping absorption bands in the general spectra, thus they are not as well differentiated as their second derivatives. This is particularly so for the NIR region as it contains overlapping combination bands and overtones of the fundamental vibrations of the MIR region. Through derivatization, the peaks can be better separated for

comparison. Hence, the second derivative HQI scores were taken into account for analysis since it would have a higher significance on the identity of the capsules.

The overall spectrum of the American white ginseng capsule was more similar to the American ginseng root than the Korean ginseng capsule to the Asian ginseng root. This was reflected by their higher correlation scores (HQI=808.85 for American white ginseng capsules, HQI=745.35 for Korean ginseng capsules). This shows that there is a wider variation among Asian ginsengs than American ginsengs since many classes of ginsengs fall under the category of “Asian ginseng”, of which Korean ginseng is but one of them. In contrast, the Korean ginseng capsule had a closer spectral similarity when its NIR spectrum was compared to its corresponding ginseng root. This was reflected in its higher mean HQI score of 684.75.

An interesting observation from the HQI scores of the capsules was that the range of scores for the American white ginseng capsules was generally wider than those for the Korean ginseng capsules. This probably led to the lower mean HQI score of the NIR spectra of the American white ginseng capsules. This was also shown in the clusters of capsules mentioned previously in Figure 4.28 in which the Korean ginseng capsule cluster was more compact. This implies that even though there are many classes of ginsengs which fall under “Asian ginseng”, there is a close similarity within each class. It has been postulated that North Korean ginseng is prepared by a secret process which involves adding certain herbs, such as with Fuzi (附子, *Aconitum carmichaeli* Debx.), to increase the potency of the ginseng and its tonics [158]. Hence even though there is a wide variation in Asian ginseng as demonstrated in earlier results, the various classes under this

broad category could probably be classified separately. However, more studies have to be done on other classes of ginseng for further confirmation.

The PCA plots comparing the capsules and the ginseng root samples are shown in Figure 4.29 (parts **a** and **b**). As shown, both types of capsules could be separated from the root samples within the first three principal components, which explained approximately 77% and 86% of their dataset variability respectively. The plots further confirmed that the similarity between the American white ginseng capsules and their corresponding root samples was higher than the Korean ginseng capsules and Asian ginseng root samples. Thus, the technique in this study was able to differentiate the ginseng capsules, as well as determine their degree of similarity to their corresponding ginseng roots, even though their properties might differ due to processing and the presence of other excipients.

American ginseng tea bags were also compared with the American ginseng root samples to provide further demonstrable proof of the sensing strategy used in this work. The American ginseng tea bags that were purchased had also claimed in its label that it contained pure American ginseng. The high HQI score of 826.2 in Table 4.3 showed that the spectra of American ginseng tea was more similar to the American ginseng root than the American white ginseng capsules, whose score was only 808.85 (Table 4.1). The possibility of the tea bags having a purer composition is high since the ginseng powder in the capsules could have been mixed with other diluents during preparation of the capsules. There was also a close resemblance in the NIR spectra of both the tea and root, as described by their mean HQI score of 792.5. Similarly, this score was also higher in the ginseng tea than the capsules (HQI=673.35).

Table 4.1. Spectral similarity between American white ginseng capsules and American ginseng roots.

American White Ginseng Capsules	HQI Scores for Spectrum Similarity			
	General MIR	2nd Derivative MIR	General NIR	2nd Derivative NIR
p1	997	930	994	935
p2	995	930	994	905
p3	992	912	992	890
p4	991	895	989	799
p5	989	892	989	797
p6	989	879	987	777
p7	988	872	987	690
p8	988	860	984	673
p9	986	858	976	664
p10	986	846	974	664
p11	983	837	970	656
p12	973	835	967	651
p13	970	829	962	624
p14	938	813	951	620
p15	937	811	935	609
p16	935	771	910	591
p17	929	726	887	588
p18	891	619	879	482
p19	883	591	867	435
p20	842	471	834	417
Average Score	959.1	808.85	951.4	673.35

Table 4.2. Spectral similarity between Korean ginseng capsules and Asian ginseng roots.

Korean Ginseng Capsules	HQI Scores for Spectrum Similarity			
	General MIR	2nd Derivative MIR	General NIR	2nd Derivative NIR
k1	967	833	995	773
k2	961	800	997	769
k3	955	800	997	769
k4	952	774	990	767
k5	949	764	979	760
k6	947	754	989	732
k7	947	748	995	720
k8	947	745	994	712
k9	946	736	994	701
k10	945	736	966	687
k11	945	734	992	679
k12	945	732	960	669
k13	943	725	991	667
k14	941	725	948	664
k15	941	724	949	663
k16	939	723	928	626
k17	939	721	952	625
k18	937	719	959	596
k19	936	716	949	562
k20	936	698	947	554
Average Score	945.9	745.35	973.55	684.75

Table 4.3. Spectral similarity between American ginseng tea and American ginseng roots.

American Ginseng Tea	HQI Scores for Spectrum Similarity			
	General MIR	2nd Derivative MIR	General NIR	2nd Derivative NIR
t1	996	981	999	840
t2	993	909	995	834
t3	992	898	994	829
t4	992	896	994	823
t5	992	873	993	823
t6	992	868	992	816
t7	989	844	992	808
t8	988	843	992	801
t9	983	837	992	801
t10	987	827	990	800
t11	987	825	990	798
t12	986	815	989	796
t13	986	804	988	793
t14	978	784	988	781
t15	980	775	987	776
t16	985	757	987	759
t17	985	752	987	755
t18	985	751	986	749
t19	982	746	986	745
t20	979	739	983	723
Average Score	986.85	826.2	990.2	792.5

Figure 4.29. 2D plots comparing commercial ginseng products with corresponding root samples. (a) American white ginseng capsules and American ginseng root samples, PC1=38% vs PC2=26% vs PC3=13%; (b) Korean ginseng capsules and Asian ginseng root samples, PC1=61% vs PC2=17% vs PC3=8%; and (c) American ginseng tea and American ginseng root samples, PC1=48% vs PC2=31% vs PC3=6%.

The first three components of their 2D plots (Figure 4.29c) accounted for up to 85% of the dataset variance. Even though the ginseng tea could be clustered, it seemed that they were separated into two groups which surrounded the American ginseng root samples. This showed that the American ginseng tea was very similar to the root in terms of their chemical constituents. This made sense as the finished tea product was purchased in the same store as the American ginseng roots. According to the retailer, the raw ginseng

roots and the ginseng that was harvested to manufacture the tea bags were from the same plot of land which the company used to cultivate the ginsengs. Thus, lesser differences would have been expected of the tea spectra as compared to the capsules. Nevertheless, the IR spectra of the samples could also be used to authenticate the American ginseng tea bags. Hence, this study shows that the IR-based analytical technique is not only useful for analyzing ginseng roots, but it can be extrapolated to the analysis of commercial ginseng products as well.

CHAPTER 5

CONCLUSIONS

Ginseng is a famous herb known for its many therapeutic effects, and its quality is important for ensuring consumer safety and efficacy. Since many ginseng products sold today are in various formulations, it is difficult to identify it by morphological means such as physical appearance, smell, or even taste. Furthermore, as ginseng is an expensive herb, adulteration with other cheaper products occurs. Thus quality assurance of ginseng is needed.

This study has reviewed the major analytical techniques that have been developed for the analysis of ginseng. The most common methods are based on chromatographic and mass spectrometric analyses of ginsenosides, which are the primary active constituents of ginseng. Among the chromatography techniques, HPLC is the most widely used and has been combined with interfaces such as ultraviolet, diode array, fluorescence and evaporative light scattering detectors. However, the detection of malonyl ginsenosides has to be done via indirect methods, since there are no malonyl ginsenoside standards.

MS techniques can also be used together with HPLC analyses to provide a confirmatory identification of the ginsenoside peaks, and tandem MS is still by far the most sensitive method for the quantification of ginsenosides. Methods of ionization and desorption include electrospray ionization, field desorption, secondary ion and electron impact ionizations. The electrospray ionization method is better than other kinds of ionization due to its high sensitivity, better reproducibility, and ability to couple with HPLC analyses. Hence, ESI-MS has become a popular method for the structural characterization of ginsenosides.

With advancement in technology, genomic and proteomic methods have also gained popularity in the analysis of ginseng. The genomic approaches are useful since adulterants can be distinguished from ginseng via different DNA sequences, while proteomic analyses give information on protein expression and can bridge the gap between their genotype and phenotype. However, genomic and proteomic analysis of ginseng is time-consuming and difficult due to the lack of DNA sequence information in ginseng populations.

For the present study, the feasibility of a novel analytical sensing strategy based on infrared wavelengths was defined for pharmaceutical ginseng products, the rationale being that TCM herbs like ginseng are complex, and the different chemical constituents in these herbs tend to exhibit characteristic IR fingerprints. Furthermore, IR spectroscopic techniques offer the advantages of being fast, non-destructive, require no or little sample preparation since they avoid tedious extraction or purification procedures, and produce no hazardous waste.

This study integrates FTIR analysis with two data processing and analytical techniques and software for the selection and determination of discriminative parameters in the original and second derivative infrared spectra of ginseng and its morphological and chemical fakes. In particular, the IRsolution 1.10[®] program (Creon Lab Control AG, Shimadzu Corporation Pte. Ltd., Asia Pacific) was used for processing of the infrared spectral data, as well as performing Pearson correlation analysis, while The Unscrambler[®] 9.2 program (CAMO software India Pvt. Ltd., Bangalore, India, Asia-Pacific) was used for principal component analysis of the spectra. The main advantages of this strategy over the conventional FTIR technique was its ability to discriminate clearly among ginsengs

and their morphological and chemical fakes by grouping them into separate clusters based on their infrared spectra, as well as determine quantitatively the degree of similarity between commercial ginseng products and the raw ginseng root.

The infrared absorption frequencies which describe the functional groups in the ginsenosides, American and Asian ginsengs are summarized in Table 5.1, and the characteristic absorption peaks in the second derivative spectra of the samples are shown in Tables 5.2 and 5.3. Based on the analytical sensing strategy defined in this work, the following conclusions were successfully obtained:

a) IR wavelengths can be used as an analytical technique for ginseng authentication. The active constituents for ginseng, known as ginsenosides, can be differentiated based on qualitative analysis, as well as PCA of their IR fingerprints. There were seven peaks in the second derivative spectra which were common and distinctive to the ginsenosides. The spectra of ginsenosides Rb₁ and Rg₁ were similar to Re and Rc respectively, and the pseudoginsenoside F₁₁ could be differentiated from the others due to the difference in its chemical structure.

Ginsengs purchased from Hong Kong could also be authenticated based on their IR spectral fingerprints. The grade 2 (G2) and grade 5 (G5) ginsengs were identified as Asian ginsengs, while the grade 10 (G10) ginseng was identified as American ginseng. However, it was noted that with more well-established and sophisticated testing methods currently available, these techniques could be used in future studies to further confirm the identities of these three grades of ginsengs. In addition, this study managed to identify the 2000-600cm⁻¹ region as the fingerprint region which could distinguish the ginsengs from each other, as well as from adulterants.

b) The two main species of ginseng can be differentiated from each other, as well as from adulterants via their spectral fingerprints. American ginseng could be differentiated from Asian ginseng via qualitative analysis of their spectral pattern differences, as well as their 2D plots. There was a wide variation in the spectra of the Asian ginsengs due to the fact that many species fall under this category, one of which was used for analysis in this study – Korean ginseng capsules.

As for the adulterants, the IR spectrum of sawdust was distinctly different from those of the ginsengs due to their differences in chemical constituents. In general, it was observed that the absorption bands in the sawdust spectrum were more prominent and distinctive than the ginsengs' spectra. The IR spectral fingerprints could also differentiate between ginseng and a chemically similar adulterant, in this case, *Platycodon grandiflorum*, also known as jiepeng. PCA of the spectra could separate the ginsengs from the adulterants as well. This was also a novel, yet interesting finding, as there have been no studies which have used PCA to distinguish ginseng from its adulterants, specifically sawdust and jiepeng. This could potentially provide a way in which ginsengs can be rapidly authenticated.

c) Commercial ginseng products can be authenticated by comparing their spectral fingerprints with those of ginseng roots. American white ginseng capsules, Korean ginseng capsules and American ginseng tea which had claimed to contain pure ginsengs were compared with their corresponding root samples. Through PC and Pearson correlation analyses, it was shown that the spectra of the products were similar to their corresponding roots. However, the American white ginseng capsules were more closely related to the American ginseng root than the Korean ginseng capsules to the Asian

ginseng root. Similarly, the American ginseng tea had a higher degree of similarity to the root than the capsules.

Table 5.1. Infrared absorption frequencies of functional groups in ginsenosides, American and Asian ginsengs.

Functional groups in ginsenosides	IR frequencies/ cm^{-1}
Alcohol OH stretch	$\sim 3333^a$
Aliphatic CH stretch	$\sim 2943 - 2893$
Carbonyl C=O stretch of CO_2	~ 2353
Alkene C=C vibrations	$\sim 1676^b$
Aromatic C-C vibrations	~ 1587
Alcohol OH bend	~ 1377
Alcohol OH stretch	~ 1072
Aryl-O- CH_3 stretch	~ 1026
Hydroxyl OH wag of water	~ 667
Functional groups in American and Asian ginsengs	IR frequencies/ cm^{-1}
Alcohol OH stretch	$\sim 3393 - 3379$
Aliphatic CH stretch	$\sim 2934 - 2928$
Carbonyl C=O stretch of CO_2	$\sim 2376 - 2315$
Carbonyl C=O vibrations in volatile oils and other compounds	~ 1735
C=C stretch of ginsenosides Rg_1 and Re	$\sim 1631 - 1618$
Amide NH bend and CN stretch	$\sim 1540 - 1523^c$
CH_3 deformations	$\sim 1411 - 1334$
C-O stretching of fats	$\sim 1102 - 1247$
C-O vibrations in alcohol hydroxyl groups	$\sim 1049 - 1024$
Ring stretching of carbohydrates	~ 927
Aromatic CH bend	$\sim 857 - 757$
Unsaturated carbon twisting and bending	~ 700

^a Peak was shifted to 3423cm^{-1} for pseudoginsenoside F_{11} .

^b Alkene band at 1676cm^{-1} was absent for pseudoginsenoside F_{11} .

^c Asian ginseng showed an absence of amide peaks.

Table 5.2. Characteristic absorption frequencies in the second derivative spectra of ginsenosides.

Characteristic IR frequencies/ cm^{-1}	Types of Ginsenosides				
	Rb ₁	Rc	Re	Rg ₁	F ₁₁
Near-infrared region					
7710					✓
7330		✓		✓	
7270	✓		✓		✓
7240		✓		✓	
7195	✓		✓		✓
5540 - 5530		✓		✓	
5370	✓		✓		✓
5300 - 5290	✓	✓	✓	✓	✓
5220	✓		✓		✓
Mid-infrared region					
1805 - 1795	✓	✓	✓	✓	✓
1685					✓
1653					✓
1353					✓
1315 - 1305	✓	✓	✓	✓	✓
1230					✓
1090 - 1080	✓	✓	✓	✓	✓
1050					✓
1030 - 1020	✓	✓	✓	✓	✓
955					✓
930				✓	
890 - 880	✓	✓	✓	✓	✓
843					✓
807					✓
740 - 730	✓	✓	✓	✓	✓
680 - 670	✓	✓	✓	✓	✓

Table 5.3. Characteristic absorption frequencies in the second derivative spectra of American and Asian ginsengs, sawdust and jiepeng.

Infrared Regions/ cm^{-1}	Characteristic IR frequencies/ cm^{-1}			
	Types of samples			
	American ginseng	Asian ginseng	Sawdust	<i>Platycodon grandiflorum</i> (Jiegeng)
Near-infrared				
7370-7050	7260 7190 7125 7075	7340 7232 7155 7106	7330 7210 7144 7078	7330 7220 7143
5490-5190	5480 5380 5297 5202	5448 5340 5245	5480 5370 5295 5203	5340 5250
Mid-infrared				
1800-1700	NA ^a	NA ^a	1752 1720	NA ^a
1600-1440	1446	NA ^a	1595 1556 1543 1509	NA ^a
1280-1260	NA ^a	NA ^a	1270	NA ^a
1100-960	1050	1073 1049 1021	1062 1027	1064 1025
900-890	NA ^a	NA ^a	895	NA ^a
700-600	606	668 609	667 625	677

^a NA, not applicable. Absorption peaks are either absent, not significant or not distinctive of sample.

Although IR spectroscopy and PCA have been used, either individually or in combination, to investigate synthetic pharmaceutical drug molecules; their combined use has not been done in the area of traditional Chinese medicine at the time of this study, particularly for analysis of ginseng quality. The turn of the century has brought with it many new discoveries, and chemometric analysis is now getting much attention in the scientific world. This study brings a whole new dimension of multivariate analysis into the world of complementary and alternative medicine.

Thus, in conclusion, the results of the present study have shown that by harnessing the power of infrared spectroscopy, PCA and Pearson correlation analytical techniques, the potential of using infrared wavelengths in the rapid analysis of ginseng quality is definitely appealing. Based on the novel “2-6 PC” rule, this study has successfully managed to show the following:

- a) Identify the $2000\text{-}600\text{cm}^{-1}$ region as the IR fingerprint for ginsengs;
- b) Differentiate American and Asian ginsengs from each other, as well as from sawdust and jiegeng; and
- c) Authenticate ginseng dosage forms even though their properties might differ due to processing techniques and the presence of other excipients.

CHAPTER 6

RECOMMENDATIONS FOR FURTHER WORK

The present work has scaled new heights in the areas of complementary and alternative medicine, as well as in multivariate statistical analysis techniques. Future work that could be done include:

- a) Determination of target wavelength markers in the IR spectra which can be used to identify or differentiate between other kinds of ginseng (e.g. Japanese, Korean, Siberian, Malaysian ginsengs);
- b) Further characterization of the constituents in ginseng based on other physical or pharmacological parameters (e.g. chirality, metabolites, pharmacokinetics, pharmacodynamics, herb-drug interactions); and
- c) Developing a novel quality sensing strategy based on current or enhanced mathematical algorithms for the detection of pharmaceutical ginseng products.

CHAPTER 7

PUBLICATIONS ARISING FROM THIS WORK

- (i) Yap, Y. L. K., Chan, S. Y., Lim, C. S., & Chan, Y. W. (2005, February 26-28). *Rapid quality surveillance of ginseng*. Paper presented at the Inaugural International Congress on Complementary and Alternative Medicines (ICCAM) 2005, Raffles City Convention Centre, Singapore.
- (ii) Yap, Y. L. K., Chan, S. Y., Lim, C. S., & Chan, Y. W. (2005, July 1-4). *Rapid quality surveillance of ginseng*. Paper presented at the 17th Singapore Pharmacy Congress, Grand Copthorne Waterfront Hotel, Singapore.
- (iii) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2005, September 16). *Rapid sensor system for ginseng identification*. Paper presented at the Inaugural American Association of Pharmaceutical Scientists-National University of Singapore (AAPS-NUS) Student Chapter Symposium, National University of Singapore, Singapore. Awarded best presenter award (2nd prize - Poster presentation).
- (iv) Yap, Y. L. K., Chan, S. Y., Chan, Y. W., & Lim, C. S. (2005). Overview on the analytical tools for quality control of natural product-based supplements: A case study of ginseng. *Assay and Drug Development Technologies*, 3(6), 683-699.

- (v) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2006, January 13). *Rapid quality surveillance of pharmaceutical ginseng biomolecules*. Paper presented at the 2nd American Association of Pharmaceutical Scientists-National University of Singapore (AAPS-NUS) Student Chapter Symposium, National University of Singapore, Singapore. Awarded best presenter award (1st prize - Oral presentation).

- (vi) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2006). Authentication of traditional Chinese medicine using infrared spectroscopy: Distinguishing between ginseng and its morphological fakes. *Journal of Biomedical Science*, doi: 10.1007/s11373-006-9133-3.

- (vii) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2006, December 11-14). *Rapid differentiation of pharmaceutical biomolecules: Ginseng and adulterants*. Paper presented at the International Conference on Biomedical and Pharmaceutical Engineering 2006 (ICBPE 2006), Orchard Hotel, Singapore.

- (viii) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2007). Infrared-based protocol for the identification and categorization of ginseng and its products. *Food Research International*, doi: 10.1016/j.foodres.2006.11.009.

- (ix) Yap, Y. L. K., Chan, S. Y., & Lim, C. S. (2006). The reliability of traditional authentication – A case of ginseng misfit. *Food Chemistry*, Submitted.

CHAPTER 8

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