

## Recent Methodology in Ginseng Analysis

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As much as the popularity of ginseng in herbal prescriptions or remedies, ginseng has become the focus of research in many scientific fields. Analytical methodologies for ginseng, referred to as ginseng analysis hereafter, have been developed for bioactive component discovery, phytochemical profiling, quality control, and pharmacokinetic studies. This review summarizes the most recent advances in ginseng analysis in the past half-decade including emerging techniques and analytical trends. Ginseng analysis includes all of the leading analytical tools and serves as a representative model for the analytical research of herbal medicines.

**Keywords:** *Panax ginseng*, Ginseng analysis, Mass spectrometry, Metabolomics, Genetic marker, Immunoassay

### INTRODUCTION

Recently, traditional herbal medicines have been gaining attention as the therapeutic candidates for many chronic or degenerative diseases caused by multifactorial causes in modern society [1,2]. Single or simple mixtures of active compounds are no longer considered to be the best therapeutic choices for treating these diseases. Formulations of natural medicines composed of diverse phytochemicals may be potential alternatives in preventing or overcoming incurable diseases through their ability to modulate multiple targets, enhance self-healing and adaptogenic capacity as described in the ethnopharmacological literature [3]. Low toxicity and reliable efficacy in humans that has been verified through a long medicinal history are other advantages of natural medicines that are encouraging the development of herbal medicines in modern therapeutics. However, the main problem in natural medicine development is quality control, as the quality can be influenced by many factors, including differences in cultivation methods, environment, and the

genetic background of plant species. Validated analytical methods for herbal medicines are important not only to establish drug standards, including species authentication and quality assessment, but also to understand drug targets and pharmacokinetic properties [4,5]. Analytical methods will therefore play an essential role in the discovery of innovative herbal medicine therapeutics.

Ginseng is a best-selling herbal medicine and has been a top-ranked subject of many fields of scientific research worldwide. The medicinal history of ginseng can be traced back over approximately 2,000 years to one of the first descriptions in ancient Oriental medical literature [6]. The English name 'ginseng' is derived from Chinese word meaning man-shaped root. Owing to this shape, ginseng is considered sacred in oriental medicine. Together with various beneficial activities, ginseng is known as a noble, miraculous medicine and is prescribed to treat many symptoms. More than 5,000 research papers have been published in authoritative scientific journals on the

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secrets of ginseng, which have traditionally been transmitted by word-of-mouth. Ginseng research must draw on aspects of natural product chemistry, pharmacognosy, pharmacology, plant bioengineering, and analytical sciences. With particular respect to analytical methodologies of ginseng, many researchers have developed methods (in terms of metabolomics) of single or multiple phytochemical analyses using sophisticated chromatographic tools as well as total extract or plant material analyses using both spectroscopic techniques and statistics [6,7]. The 'ginseng analysis' should be the professional term used to refer to analytical approaches for ginseng, and is a representative model for other natural medicines.

In this review, we summarize the advances in ginseng analysis over the past 5 years with a brief overview on the background of ginseng, such as the botanical species, types of ginseng, chemical constituents, and pharmacology. The various analytical methods are the main focus, including separation-based single/multiple phytochemical analysis, whole mixture analysis without separation, genetic techniques, and immunoassays. This review will provide a comprehensive overview of the current trends in ginseng analysis and give new insights into the future of analytical techniques for natural medicines.

## BOTANICAL SPECIES OF GINSENG

Traditionally, 'ginseng' refers to the root of *Panax ginseng*. However, plants belonging to the genus *Panax* have recently also been denoted as 'ginseng'. Ginseng is also named after its native habitat, such as Korean, Chinese, American, and Japanese ginseng. Of these, ginseng (Korea) has been studied the most intensively. It is *P. ginseng* Meyer native to Korea and is the most well-known ginseng with potent pharmacological efficacies [8]. Chinese ginseng is *P. notoginseng* (Burk.) F. H. Chen native to China. American ginseng is *P. quinquefolius* L. native to North America, including Canada and United States. These 3 species are widely used as a functional food and in traditional medicine. In addition, *P. japonicus* Meyer (Japanese ginseng), *P. pseudoginseng* subspecies *Himalacius* (Himalayan ginseng), *P. trifolius* (dwarf ginseng), and *P. vietnamensis* Ha et Grushv. (Vietnamese ginseng) are lesser known *Panax* species [7]. However, adaptogenic plants in some countries are called ginseng owing to their ginseng-like effects. Because these plants are not the *Panax* species, they are not considered to be true ginseng. These ginseng alternatives include, among others, Siberian ginseng (*Eleutherococcus senticosus*), Indian ginseng (*Withania somnifera*), and Brazilian ginseng (*Pfaffia paniculata*).

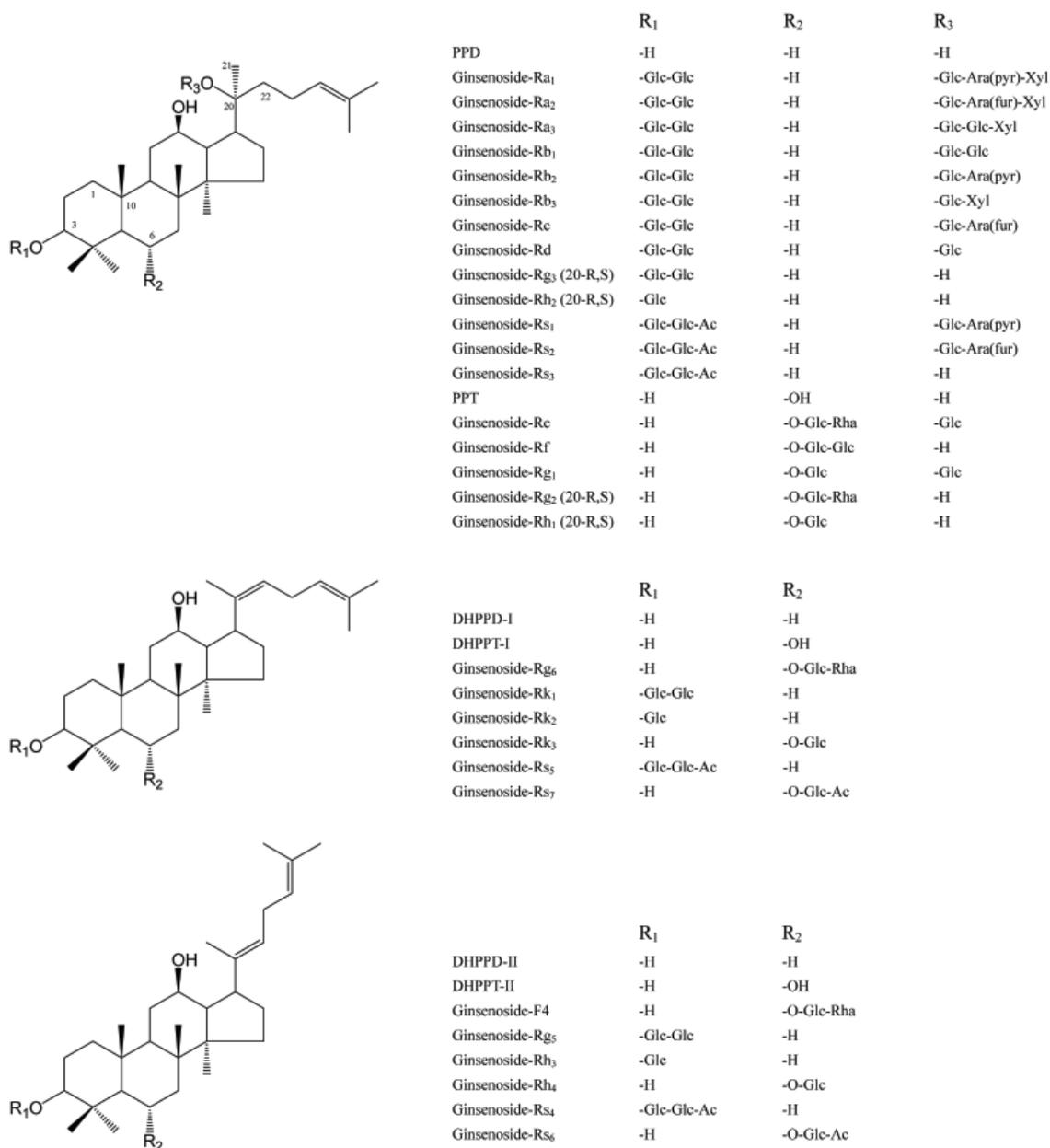
## TYPES OF GINSENG PREPARATIONS

Ancient pharmacists created transformed herbal medicines through steaming, roasting, or fermenting. Processing leads to changes in the component phytochemicals, thereby expanding the chemical diversity and bolstering the overall efficacies [9]. Processing is now gaining interest in an effort to create new medicinal recourses having diverse chemical pools, and many new advanced techniques are currently being examined and applied.

Traditionally, ginseng refers to the root, and the other parts of ginseng, such as the leaves and berries, are rarely used. The ginseng preparations described below follow this traditional notion. Fresh ginseng refers to immediate harvest without any additional processing. Because of problems during storage or circulation, in most cases, fresh ginseng is turned into white or red ginseng. White ginseng refers to dried ginseng, while red ginseng is the transformed ginseng created by traditional processing with successive steaming and drying. Our research group developed a new processed ginseng (called sun ginseng) from *P. ginseng* through steaming under optimized high temperature and pressure conditions. Sun ginseng contains increased levels of biologically active, less polar ginsenosides and enhanced pharmacological activities compared to those of white and red ginseng [10,11]. This processing method has also been applied to different *Panax* species and other natural medicines [12]. Other types of processed ginseng have been developed using modified steaming, explosive puffing, or fermentation with specific microorganisms, such as intestinal microbial flora. Black ginseng is prepared by repeated cycles of atmospheric steaming and sun-drying [13]. Puffed ginseng is prepared by applying an optimized puffing pressure and residual moisture content in ginseng [14]. Fermentation with intestinal microbial flora leads to structural modification of the ginsenosides. Compound K, the intestinal metabolite of ginsenoside, is a representative bioengineered ginsenoside that shows potent anti-cancer effects [15]. New types of processed ginseng preparations will be developed in the future [16]. Aside from the ginseng preparations that have originated from cultivated ginseng plants, wild mountain ginseng grown in the highlands is also available.

## PHARMACOLOGICAL EFFICACIES AND CHEMICAL CONSTITUENTS OF GINSENG

As the genus name *Panax*, meaning 'cure all' in Greek, implies, ginseng has been referred as a panacea and pre-



**Fig. 1.** Chemical structure of ginsenosides in *Panax ginseng*. PPD, protopanaxadiol; PPT, protopanaxatriol; DH, dehydro; Glc, glucose; Ara, arabinose; Xyl, xylose; Rha, rhamnose; Ac, acetyl; pyr, pyranosyl; fur, furanosyl.

scribed for many serious symptoms. Ginseng is basically a tonic or adaptogenic herb that improves the basal level of health, body balance, and restoration capacity. Beyond its health-improving and self-healing functions, the pharmacological activities of ginseng span a broad range of protections to direct therapeutic effects on various organs and diseases. Because an enormous quantity of research has been conducted to uncover the biological activities of ginseng, all individual activities could not be listed in the limited space of this review. In this regard, the many

reviews must serve as thorough guides to explore the pharmacology of ginseng [8,17-19].

Numerous compounds, from small molecules to macromolecules, have been isolated from and identified in ginseng, including triterpenesaponins, polyacetylenes, alkaloids, phenolics, polysaccharides, peptidoglycans, and proteins. Ginsenosides, the triterpene glycosides (saponins), are the unique and active constituents in ginseng. More than 100 ginsenosides have been isolated from all parts of the *Panax* species. Fig. 1 shows the

structure of ginsenosides isolated from the *P. ginseng*, red ginseng, and sun ginseng. Protopanaxadiol and protopanaxatriol are the main structural features of the aglycone moiety of ginsenosides in *P. ginseng*. Excessive steaming results in modification of the ginsenoside structure, characterized by deglycosylation of the sugar moieties and double bond formation ( $\Delta^{20(21)}$  or  $\Delta^{20(22)}$ ) following dehydroxylation at C20. Less polar ginsenosides with this aglycone structure are the unique and biologically active ingredients in red ginseng and sun ginseng [10]. Finally, polysaccharides are the other major and biologically interesting constituents in ginseng. Many polysaccharides, including panaxans, ginsenosans, and pectins, have been isolated from ginseng and characterized. Ginseng polysaccharides show anti-diabetic, immune-modulating, anti-cancer, and anti-infection activities [20].

## ANALYTICAL METHODS

Recent analytical methodologies are reviewed below on the basis of key techniques or analytical instrumentation. Most methods are separation-based tools, including TLC, HPTLC, HPLC, UPLC, GC, MS, capillary electrophoresis (CE), and counter current chromatography (CCC). Methods that do not rely on separation are also described, including metabolomic fingerprinting (metabolomics), genetic marker analysis, and immunoassays.

### Thin layer chromatography and high-performance thin layer chromatography

TLC is a basic chromatography technique generally used for the quick identification or characterization of analytes. Because of its ease of use, versatility, high speed, and low cost, TLC is an excellent tool for the drug identification described in pharmacopoeias and is still used today in the pharmaceutical industry. Using uniform and fine coating materials (5  $\mu\text{m}$  of mean particle size), HPTLC yields higher separation, resolution, and reproducibility than conventional TLC. Moreover, a densitometric analysis of colored spots on TLC plates enables the quantitation of ginsenosides in crude or formulated ginseng preparations [21]. Recently, Reich *et al.* [22] validated an HPTLC method for the identification of botanical materials. This method is used for fingerprinting the ginsenoside composition in many *Panax* plants, including *P. ginseng*, *P. quinquefolium*, and *P. notoginseng*, and is useful as an official method in a current Good Manufacturing Practices environment.

### High-performance liquid chromatography and ultra-performance liquid chromatography with hyphenated detection techniques

In ginseng analysis, HPLC is coupled with many different kinds of detectors, including UV detector, diode array detector (DAD or photodiode array detector), evaporative light scattering detector (ELSD), charged aerosol detector (CAD), pulsed amperometric detector (PAD), and MS [6,23]. Most MS applications will be discussed in a separate section.

HPLC is a highly favored and general method for ginseng analysis. It is versatile for most analytes, easy to use, accurate, and precise. Generally, most HPLC columns used for ginseng analysis are reversed-phase and packed in a standard 150 or 250 $\times$ 4.6 mm column with 5  $\mu\text{m}$  silica beads that are covalently coated with  $\text{C}_{18}$  sorbents. Acetonitrile and buffered water are the typical solvent systems of choice for the mobile phase. Different stationary phases, including normal-phase, ion-exchange, and hydrophilic interacting materials, are also used for specific purposes [6,7,23]. Modified sorbents, such as polar end-capped materials or ultrafine sorbents less than 5  $\mu\text{m}$  in size, are also available and present potential ways to improve HPLC analysis and applications.

Most HPLC applications in ginseng analysis over the past 5 years have involved the quantitation of ginsenosides or related projects [24]. Despite the frequent use of UV or DAD due to their ease of use or the ability to collect analytes after separation, these detectors have many weaknesses in ginsenoside analysis, as ginsenosides absorb UV rather poorly. UV detection of ginsenosides is usually performed at a short wavelength of 198 to 205 nm, which creates a noisy baseline and limits the choice of solvents and modifiers. Photodiode array detectors, also called DADs, can provide real-time spectra from several wavelengths simultaneously. However, the sensitivity of a DAD is lower than that of a single wavelength UV detector that uses photomultiplier tubes to convert light into electrical signals. Using UV or DAD, ginsenosides have been determined from various *Panax* species and ginseng preparations [25-29]. Both UV and DAD could be easily connected to other types of detectors such as ELSD or MS. The simultaneous detection of UV-ELSD enabled the sensitive analysis of multiple components in natural medicine formulations containing ginseng, including UV- or ELSD-amenable [30,31]. Aside from ginsenosides, polyacetylenes have also been determined by UV detection in American ginseng roots [25,27]. Digitoxin and digoxin are useful internal standards in ginsenoside analysis with HPLC-DAD-MS or

HPLC-ELSD [32,33]. A principal component analysis of data sets obtained from HPLC-DAD chromatograms is useful for distinguishing adulterations from different *Panax* plants in ginseng formulations [34]. In addition to ginseng saponin analysis, Qian *et al.* [35] used HPLC-UV for the simultaneous determination of nucleosides in *P. notoginseng* using a multiple column switching technique.

ELSD is a promising alternative that could overcome the limitations of UV detection in ginsenoside analysis. The main problems of UV detection of ginsenosides are a noisy baseline and limited choice of solvents and modifiers due to the poor UV absorption by ginsenosides. ELSD is a universal and mass-selective detector for non-volatile compounds that produces a stable baseline, even with gradient elution. Furthermore, many volatile mobile phase modifiers are available to obtain better resolution and selectivity [6,23]. Our research group was the first to use ELSD to quantify ginsenosides on an amino-bonded column [36]. We also reported an optimized HPLC-ELSD method using a reversed-phase column for the determination of ginsenosides in white, red, and sun ginseng [11]. Since then, ELSD has been considered the standard choice for ginsenoside analysis. The HPLC-ELSD method has primarily been used for the simultaneous quantitation of various ginsenosides from *Panax* species or transformed ginseng preparations [33,37-40], and a principal component analysis of HPLC-ELSD chromatograms has been used to discriminate the geographic origins of *P. ginseng* roots [41]. Most ELSD analyses produce good chromatographic parameters, including high precision and accuracy, and the limit of detection (LOD) for ginsenosides has been determined at approximately 100 ng [37,38].

CAD was developed as an alternative to ELSD to detect poor UV-responsive analytes. CAD is a mass-selective detector for non-volatile compounds, and its major advantages over ELSD are enhanced sensitivity and reproducibility. Using an HPLC-CAD system, Bai *et al.* [42] and Wang *et al.* [43] simultaneously quantified ginsenosides in *P. ginseng* and *P. notoginseng*. They found that CAD produced improved chromatographic parameters, including sensitivity, linearity and reproducibility, over UV and ELSD. The LOD and limit of quantitation (LOQ) for ginsenosides ranged from 0.01 to 0.15 µg and 0.04 to 0.41 µg, respectively [42]. We anticipate that the use of CAD will continue to increase in the near future in ginseng analysis as an alternative to UV and ELSD.

PAD is a less popular HPLC detector in ginseng analysis. It is an electrochemical detector that measures

the positive potential produced by sample oxidation on a gold electrode, lending itself to carbohydrate or poly-alcohol quantitation coupled with high-performance anion-exchange chromatography (HPAEC). Our research group was the first to develop an ion chromatography-PAD method for ginsenoside analysis [44]. Joo *et al.* [45] simultaneously determined two Amadori compounds (arginyl-fructose and arginyl-fructosyl-glucose) in Korean red ginseng and plasma samples from rats treated with red ginseng extract using HPAEC-PAD. Finally, Kwon *et al.* [46] and Kwon *et al.* [47] developed a method for the highly sensitive quantitation of ginsenosides using reversed-phase HPLC-PAD under alkaline conditions. Their PAD method produced excellent linearity and improved LOD of less than 0.1 ng for non-polar ginsenosides.

More recently, UPLC, an advanced type of HPLC, has emerged as a powerful tool in many analytical laboratories to profile phytochemicals in crude plant extracts [48]. UPLC uses short, narrow-bore columns packed with sorbents that is less than 2 µm in size, thereby permitting faster analysis times and ultra-high resolution [6]. Existing HPLC conditions can be directly transferred to UPLC with only slight modifications. The UPLC-DAD method has been used for the rapid and simultaneous determination of saponins in *Panax* plants [49,50]. The LOQ and LOD (0.2-2.4 ng and 0.1-1.8 ng, respectively) were dramatically improved over the conventional HPLC-DAD method. UPLC minimizes the running volumes of organic solvents as the mobile-phase, improving MS accessibility by minimizing the burden on the MS interfaces, that move ions from solution into the gas phase before entry into the MS. Dan *et al.* [51] and Xie *et al.* [52] developed an UPLC-electrospray (ESI)-MS method to determine saponins in *P. notoginseng*, and an UPLC-time-of-flight (TOF)-MS for metabolomics profiling of several *Panax* plants. Additional UPLC-MS applications will be discussed in the mass spectrometry section.

In addition to the aforementioned techniques, several other variations of LC methods have been reported. Rapid resolution LC with a short analytical column packed with ultrafine sorbents was used for the rapid and simultaneous determination of ginsenosides in *Panax* plants [53]. A radioactive tracer assay coupled with HPLC was used for the pharmacokinetic profiling of radioisotope labeled-ginsenoside Rd [54]. Using an HPLC system, Hu *et al.* [55] studied the retention behavior of ginsenosides on a reversed-phase stationary phase in varying conditions of solvent, pH, ionic strength, and column temperature. Using hydrophilic interaction chromatog-

raphy, Quiming *et al.* [56] studied a retention prediction model system for ginsenosides on a polyamine-bonded stationary phase. Using high-performance size exclusion chromatography and HPLC, Guan *et al.* [49] suggested a method for distinguishing polysaccharides in many natural medicines by saccharide mapping following the chromatographic analysis of enzymatic hydrolysates. Li *et al.* [57] developed an effective purification method for ginsenosides from ginseng preparations using AB-8 macroporous adsorption chromatography, Amberlite IRA 900 CI anion-exchange chromatography, and Amberlite XAD16 adsorption chromatography. Finally, Kuang *et al.* [58] developed a preparative purification procedure for ginsenoside Re from the ginseng bud using selective adsorption onto an activated carbon column.

As described above, HPLC and related chromatographic tools have been widely used in ginseng analysis. We anticipate that the HPLC method will continue to be improved upon and used in the future along with technological developments in analytical instrumentation and separation media.

### Gas chromatography with hyphenated detection techniques

GC is a high-resolution and, environment friendly separation technique that avoids the use of toxic solvents. Its main disadvantage is its limited application to volatile compounds. Silylation (i.e., trimethylsilyl-derivatization) involves the substitution of the active hydrogen on a compound with an alkylsilyl group, such as a trimethylsilyl (-SiMe<sub>3</sub>). Because the silylated derivatives are less polar, more volatile, and more thermally stable, they can be easily separated by gas chromatography and related techniques. Christensen summarized the silylation strategies for measuring ginsenoside content with GC after the hydrolytic conversion of ginsenosides to their corresponding aglycon [59]. Recently, Xie *et al.* [60] determined dencichine, a neurotoxic non-protein amino acid in *Panax* plants, using ethyl chloroformate derivatization followed by GC-MS.

Volatile compounds in ginseng have easily been determined by GC with applicable detection technologies, such as MS. Xie *et al.* [61] determined volatile oils (e.g., alpha-cadinol, alpha-bisabolol, thujopsene, and n-hexadecanoic acid) in the root of *P. ginseng* at different ages. Abd El-Aty *et al.* [62] characterized volatile flavor compounds with GC-MS and determined their content in white and red ginseng. Finally, Liu *et al.* [63] determined polyacetylenes in *Panax* species using GC-MS method.

Another important application of GC is in the analysis

of pesticides and their metabolites in ginseng samples. Pesticides are exogenous chemicals that are unavoidable in the cultivation of ginseng. Because the regulation of pesticides is different in different countries, pesticide profiling could provide critical clues in tracking the geographical origins of ginseng samples and involving safety issues for the consumption of ginseng preparations. GC has been coupled to several different detectors for the simultaneous quantitation of organohalogen and organophosphorous pesticides. While the electron capture detector and nitrogen-phosphorous detector are the classical detection techniques [64,65], flame photometric detector and many types of MS detectors, including single/triple quadrupole and time-of-flight analyzer, are being used with automated sample handling systems [66,67]. New sample preparation techniques or isotope dilution method are also being studied with ginseng samples [68-70], and the official protocol for minimizing the inter-laboratory variation in pesticide profiling is still at the initial stage of consideration [71]. Additionally, HPLC has also been used for the simultaneous determination of pesticides [72].

### Mass spectrometry

MS-based applications have occupied a major portion of ginseng analyses in recent years and are leading the trend in analytical methods. Because of its powerful identification and quantitation capabilities, MS is a versatile analyzer for the analysis of non-volatile molecules in ginseng, such as ginsenosides and their metabolites in biological systems [6,7,23]. MS is mostly used as a hyphenated detector coupled to chromatography instruments, such as HPLC, UPLC, and GC. Less common is direct sample analysis without separation using a direct insertion probe for volatile ginseng compounds or flow injection techniques for mass spectroscopic fingerprinting of *Panax* species [62,73]. As a liquid chromatography detector, an interface module with an ion source is generally used to evaporate solvents and convert sample molecules in the gas phase into ions or to move ions from the solution to the gas phase. Our group developed a thermospray LC-MS method for ginsenoside analysis [74]. However, the thermospray interface is currently used less frequently. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are typically used in ginseng analysis, with ESI being more common [75,76]. Electron impact (EI) ionization is the most commonly used GC-MS technique. While ESI and APCI are known as soft ionization methods that conserve the molecular ion, EI is a hard ionization technique that

produces heavy fragmentation. Matrix-associated laser desorption/ionization is a soft ionization technique, allowing for the analysis of macromolecules such as biomolecules and organic polymers. It is used for the mass spectrometric imaging of ginsenoside localization in *P. ginseng* root [77].

Various types of mass analyzers are used for ginseng analysis: quadrupole (Q), ion trap (IT), TOF, and Fourier transform ion cyclotron resonance (FT-ICR) [6]. Moreover, multi-stage MS (also called tandem MS or MS/MS or MS<sup>n</sup>) combines different mass analyzer designs that give advanced structural information, sensitivity, specificity, and versatility [6]. Triple quadrupole (QqQ), Q-IT, Q-TOF, or IT-TOF are used for ginseng analysis.

Quadrupole is the simplest and least expensive mass analyzer. Li *et al.* [78] and Li *et al.* [79] studied the pharmacokinetic profiles of *P. notoginseng* by determining multiple saponins in rat plasma simultaneously using HPLC-ESI-MS (single Q analyzer). The IT is an advantageous mass analyzer capable of single and multi-stage MS. Ginsenosides and its metabolites were characterized and determined by HPLC-MS (IT analyzer) [80,81]. TOF analyzer measures accurate mass with high resolution and a full-scan mass range, thereby making it suitable for the identification of unknown metabolites. However, a narrow linear range of quantification and expensive instrumentation are the main disadvantage of TOF compared to Q and IT analyzers. Chen *et al.* [80] developed a rapid and sensitive analytical assay to determine multiple bioactive constituents in ginseng preparations using LC-ESI-TOF. Most TOF analyzers are coupled to UPLC for ginseng analysis and are discussed in greater detail later. An FT-ICR, the most expensive mass analyzer, can be used as a multiple-stage MS similar to IT and also has a wide mass range and high mass resolution similar to TOF. Kong *et al.* [82] developed an HPLC-FT-ICR method for identifying the gastro-intestinal metabolite of ginsenosides *in vitro*.

HPLC-tandem MS applications have frequently been used to determine bioactive, new, or transformed phytochemical profile in many *Panax* plants or ginseng preparations [83-86]. They have also been used for the pharmacokinetic profiling of ginsenosides in biological systems [87-92]. HPLC-MS is also useful to study interactions between drugs and other herbal medicines with ginseng [93,94]. Both UV and mass profiles obtained by HPLC-DAD-MS analysis for chemical fingerprinting and quantitative analysis can be used for species authentication or quality control [95-97].

UPLC has drawn huge attention as a high-resolution

chromatography system. Because UPLC provides high resolution and high accessibility to MS, it is generally used in MS-based metabolomics. Metabolomic studies of raw and steamed *P. notoginseng* have employed UPLC-TOF [68,98]. Dan *et al.* [51], Xie *et al.* [52], and Dan *et al.* [99] applied various MS systems, such as UPLC-ESI-TOF and UPLC-ESI-QqQ, for saponin and metabolite profiling in *Panax* plants. Pharmacokinetic studies have used UPLC-MS or tandem MS to simultaneously determine various ginsenosides [100,101]. In addition, UPLC/Q-TOF has been used for the rapid identification of adulterated ginseng preparations, chemical profiling of decoction-induced chemical transformation in ginseng preparations, and bioactive ginseng saponin identification [102-105].

### Capillary electrophoresis

CE, also known as capillary zone electrophoresis, can be used to separate electrically charged analytes based on their size-to-charge ratio in the interior of a small capillary filled with an electrolyte. Because most ginsenosides are neutral molecules, conventional CE could not separate them. However, modified CE techniques are used for ginsenoside analysis, such as micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC). Both MEKC and MEEKC can separate analytes by their differential partitioning between water-immiscible particulates (micelles and microemulsion) and a surrounding aqueous buffer solution.

Cao *et al.* [106] are the leading group developing MEKC and MEEKC methods for ginsenoside analysis. They developed and optimized an on-line injection technique for the analysis of neutral ginsenosides (ginsenoside Rg<sub>1</sub>, Rf, Rh<sub>1</sub>, Rd, Rg<sub>3</sub>, and notoginsenoside R1) with maximum stacking efficiency for good separation in MEEKC. They also developed a MEKC method with nonionic Brij-35 micelles that utilized on-line dual sweeping based on borate complexation and enhancement of the organic solvent field for the preconcentration of neutral ginsenosides [107]. Subsequently, they established a complex formation and acetonitrile sweeping technique for non-ionic MEEKC with UV detection for ginsenosides Rf, Rb<sub>2</sub> and Reusing a Brij-35 microemulsion [107].

### Counter current chromatography

CCC is a liquid chromatography technique that separates solutes based on their partitioning between 2 immiscible liquid phases, usually under centrifugal force.

Advanced types of CCC with hyphenated detectors such as ELSD have been used in ginseng analysis: high-performance CCC, high-speed CCC, and centrifugal partition chromatography. These analytical instruments were primarily used for the preparative isolation or purification of selected ginsenosides, including ginsenoside Rb<sub>1</sub>, Rd, Re, Rf, Rg<sub>1</sub>, Rg<sub>3</sub>, Rg<sub>5</sub>, Rk<sub>1</sub>, F4, and notoginsenoside R1 [108-110].

### Metabolomic fingerprinting and metabolomics

Conventionally, multivariate statistical analysis of spectral datasets has been used for the authentication or relevant grouping of plant species, rapid evaluation of composition, quality control, and metabolomics [6,7]. This section illustrates the rapid analytical methods used for ginseng without any separation procedures and the advanced statistics for data analysis.

NMR-based metabolomics are the most frequently used for ginseng analysis. These approaches are primarily aimed to discriminate ginseng samples by age, species, and cultivation area [111-115]. Lee *et al.* [116] suggested NMR-based metabolomics as a new quality assessment tool for ginseng. Major ginseng metabolites, including amino acids, have been identified as promising biomarkers for quality assurance in ginseng. NMR-based profiling is simple, comprehensive, and reproducible. However, low sensitivity for minor components remains a limitation of this method.

Infrared (IR) and Raman spectrometry are less frequently used for ginseng analysis relative to NMR. However, IR has been applied to species authentication and as a quality control in the industry. IR and Raman spectroscopy are simple and fast, but not applicable to quantitation. Most IR analyses have been used to generate spectroscopic ginseng datasets for ultimate metabolomics fingerprinting with multivariate statistical analysis. Yap *et al.* [117] established an IR-based fingerprinting method to detect and authenticate ginseng in many products [117]. They also suggested a “2-6PC rules” rapid and effective method for authenticating ginseng and discriminating between different *Panax* species [118]. In addition, Fourier transform infrared (FTIR) spectroscopy, 2D-FTIR and 2D-IR correlation spectroscopy, have been introduced for species identification, authentication, and the differentiation of age and cultivation origin [119-122].

Fourier transform Raman (FT-Raman) spectroscopy has alternatively been used for the same purpose as other fingerprinting techniques. Edwards *et al.* [123] achieved analytical discrimination of ginseng sources with FT-Raman spectroscopy. Chinese ginseng had unique Raman

spectral features at 980 cm<sup>-1</sup>.

Other methods include light stable isotope analysis or strontium isotope analysis, which was used to differentiate between different geographical origins of ginseng [124,125]. The δ<sup>2</sup>H ratios were significantly different in ginsengs cultivated in Korea or China. Multivariate statistics are essential to fingerprinting techniques. Principal component analysis has been frequently used as a general statistical tool. Han *et al.* [126] suggested score-moment combined with linear discrimination analysis to improve discrimination efficiency. This method was well suited for the Raman spectroscopic discrimination of ginsengs cultivated in Korea and China.

### Genetic marker analysis

A genetic marker is a gene or DNA sequence with a known location on a chromosome that could be used to identify a species. Genetic marker analysis has primarily been used to authenticate individual *Panax* species or cultivars, thereby contributing to the detection of specific ginsengs and detecting adulteration in commercial ginseng products.

Many genetic markers or tools have been examined in recent genetic analyses of ginseng. Genetic markers for ginseng include simple sequence repeats (also called microsatellites) [127,128], randomly amplified polymorphic DNA [129], restriction fragment length polymorphisms [130,131], insertion/deletion markers [132], single nucleotide polymorphisms (SNPs) [132], amplified fragment length polymorphisms (AFLPs) [133], AFLP-derived sequence characterized amplified regions [134], and cleaved amplified polymorphic sequence markers [135]. Novel genetic tools to discover markers have also been developed including loop-mediated isothermal amplification [136] and DNA microarray-based fingerprinting [137,138].

Sathiyamoorthy *et al.* [139] and Wang *et al.* [140] performed a transcriptomic analysis of ginseng roots to study the expressed sequence tags putatively involved in the ginsenoside biosynthetic pathway and stress resistance. Their results may contribute to the genetic engineering of ginseng plants, such as the development of stress-resistant and ginsenoside-rich plants. They also discovered new genetic targets for determining the new ginseng cultivar “Chunpoong”, such as a major latex-like protein gene [132], mitochondrial nad7 intron 4 region [141], and mitochondrial cytochrome oxidase subunit 2 intron I/II regions [142]. Together, they suggested the molecular identification of the ginseng cultivar “Gumpoong” based on 26S rDNA analysis [143], and

“Chunpoong” or “Yunpoong” cultivars based on real-time PCR-based SNP marker analysis [144-146]. Analysis of SNP markers may contribute to the molecular authentication of *P. ginseng* and products [147]. More recently, Zuo *et al.* [148] presented a DNA barcoding method for the identification of diverse *Panax* species and clusters through the combinatorial analysis of both the PSBA-TRNH (encodes photosystem II protein D1-tRNA<sup>His</sup>) noncoding region and the internal transcribed spacer region. Genetic marker analysis provides direct evidence for species authentication, the identification of adulteration, and genetic information regarding specific bioactive component biosynthetic pathways [149-152]. Along with the development of molecular biotechnology, innovative genetic tools will likely provide promising options for the identification of ginseng species at the DNA level.

### Immunoassay

Immunoassays utilize the specific reaction between an antibody and its antigen for qualitative or quantitative analysis. Recent immunoassays used in ginseng analysis include the enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunofluorescence. ELISA is a specific and accurate quantitation assay in which the antibody is conjugated to an enzyme, such as horseradish peroxidase. Pongkitwitoon *et al.* [153] and Sritularak *et al.* [154] used a monoclonal antibody against specific ginsenosides for the ELISA-based determination of ginsenoside Rb<sub>1</sub>, Rg<sub>1</sub>, and Re in American ginseng berries and flowers [153,154]. The development of antibodies targeting ginsenoside has also contributed to the preparation of knockout extracts using immunoaffinity chromatography [155,156]. Meanwhile, Morinaga and Shoyama [157] and Tanaka *et al.* [158] developed a new immunoblotting method called “Eastern blotting”. Ginsenosides, pre-separated on a silica gel TLC plate, were transferred to a polyvinylidene fluoride membrane that was treated with NaIO<sub>4</sub>, which was then treated with bovine serum albumin (BSA) to form a ginsenoside-BSA conjugate. A monoclonal antibody targeting aglycon of ginsenoside was used for immunodetection. Quantitation was performed by image-based analysis of color density, following immunodetection and corresponding substrate treatment. This “Eastern blotting” method was used to determine ginsenoside Rb<sub>1</sub>, Rg<sub>1</sub>, and Re with monoclonal antibodies targeting each ginsenoside [158,159]. Moreover, the localization of ginsenoside Rb<sub>1</sub> in the various parts of *P. ginseng* was monitored by immunofluorescence with a monoclonal antibody targeting ginsenoside Rb<sub>1</sub> [160].

## CONCLUSION

Ginseng has served as a model not only for testing the performance of various analytical techniques but also for developing novel analytical methods to characterize natural medicines. Recent methodologies employed in ginseng analysis range from single/multiple component quantitation to fingerprinting or metabolomics with statistical analysis. Chromatography with hyphenated detection, mainly HPLC, has still been the primary method of ginseng analysis. UPLC with analytical column technology enables fast separation and high resolution. Sophisticated MS instrumentation guarantees ultra-high accuracy, precision, and sensitivity. Advanced multivariate analysis has also accelerated the development of informatics and metabolomics. Biochemical approaches, such as immunoassays and genetic marker analyses, are being used with increasing frequency in ginseng analysis and are likely to contribute more significantly in the future. Relevant technical advances may help in understanding the *Panax* species, thereby contributing to the creation of beneficial bioengineered species. The major applications of ginseng analysis are in species authentication, quality control, pharmacokinetic profiling, and biomarker identification in biological systems. A comprehensive understanding of individual approaches and accumulated applications in the laboratory and clinics may provide promising insights for further development of ginseng analysis.

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