

**SPECIAL FEATURE:
PERSPECTIVE**

A capsule review of recent studies on the application of mass spectrometry in the analysis of Chinese medicinal herbs

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Chinese herbal medicine is gaining increasing popularity worldwide as an alternative approach to the development of pharmaceuticals in therapeutic applications. Chemical characterization and compositional analysis of Chinese medicines provide the necessary scientific basis for the discovery and development of new drugs of natural origin. Applications of mass spectrometry in the analysis of Chinese herbal medicines have been growing rapidly in recent years owing to the rapid technical advances and increasing availability of the instrumentation. This paper reviews the current status of how different mass spectrometric techniques are being used to support research studies of Chinese medicines. The focus is on crude herbal medicines and their derived products. The review is not meant to be exhaustive, but rather to provide a general overview of the various research activities in this rapidly expanding field. In the discussion of specific herbs, the emphasis is placed on ginseng and *Danshen*, two of the herbs for which active experimental work is on-going in the authors' laboratories. Other selected herbs will be discussed only briefly, aiming primarily to illustrate the current status of research in the area. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: Chinese medicinal herbs; ginseng; *Danshen*; mass spectrometry

INTRODUCTION

Chinese medicine has been the most organized traditional medicine in the world. It has been used for the treatment of diseases for thousands of years. Throughout China's long history, Chinese people have accumulated a rich empirical knowledge of the properties and usage of natural products and an enormous body of human clinical data on their efficacy and toxicity. The therapeutic effects and minimum side effects of many herbal remedies have recently been demonstrated or verified in numerous modern scientific investigations. These therapeutic effects are often

complementary to those of Western drugs, which explains the recent burst of enthusiasm worldwide on the study of traditional Chinese medicines. The search for 'miracle cures' for serious disease such as cancer and AIDs, for example, has further fueled the growing interest in herbal Chinese medicines.

Although many Chinese medicines are effective in treating diseases, their remedial mechanism is not well understood. The analysis of active components in Chinese medicinal extracts is a key to unlocking the secret of their effectiveness. The major compound types in Chinese medicinal herbs include alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins. Efficient detection and rapid characterization of these components on a molecular basis play an increasingly important role as analytical support in scientific studies aimed at a better understanding of the pharmacological basis of Chinese herbal medicines. The usual approach to such studies involves the preparation of herbal extracts, testing their pharmacological activity, isolating the individual components of the extracts by using liquid chromatography (LC), and then performing structure elucidation by nuclear

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magnetic resonance (NMR), mass spectrometry (MS) and other spectroscopic techniques. Among the latter structural analysis techniques, applications of MS in Chinese medicinal research have been accelerating in recent years because of the rapid developments in MS and associated techniques. The capability of MS to perform on-line compositional and structural analyses through the use of hyphenated techniques such as GC/MS, LC/MS and MS/MS provides rich information at a rapid rate, which is unsurpassed by other techniques. A significant number of reports have recently been published on the determination of active ingredients in the extracts of Chinese herbal medicines utilizing mass spectrometric approaches.

APPLICATIONS OF VARIOUS MASS SPECTROMETRIC TECHNIQUES

Reports on the applications of mass spectrometry in Chinese medicinal analysis have been increasing rapidly in recent years. Different ionization methods for these mass spectrometric analyses have been published, including electron ionization (EI),^{1–17} field desorption (FD) ionization,^{18,19} ²⁵²Cf-plasma desorption,²⁰ fast atom bombardment (FAB),^{21–25} matrix-assisted laser desorption/ionization (MALDI),^{26,27} thermospray²⁸ and electrospray ionization (ESI).^{29–44} Mass spectrometry, coupled with chromatographic separations such as gas chromatography (GC/MS) and liquid chromatography (LC/MS), is used for direct analysis of components existing in traditional Chinese medicines. Mass spectra of various components in sample extracts of Chinese medicines have been obtained and matched against known standards for structural confirmation. Consequently, GC/MS, LC/MS and MS/MS fingerprinting profiles of the active ingredients were collected and information is starting to be built up for a wide variety of herbs and their derived products.

GC/MS has been demonstrated to be a valuable analytical technique for the analysis of non-polar components and volatile essential oils extracted from Chinese herbal plants.^{2–17} Chen *et al.* in 1987 described a method using direct vaporization GC/MS to determine approximately 130 volatile constituents in several Chinese medicinal herbs.² The direct vaporization results were found to agree well with those obtained by traditional steam distillation extraction methods. Cairns *et al.* demonstrated that preliminary screening of Chinese herbal medications can be accomplished by a combination of TLC and GC/MS.³ GC/MS was applied to analyze fatty acids and lipids in Chinese medicinal seed oils by Jie and co-workers.^{4,5} Betz *et al.* used chiral GC to separate and determine ephedrine-type alkaloids in dietary supplements containing *Mahuang*.⁸ The identities of the alkaloids were verified by GC/MS and GC/FTIR. Hou *et al.* reported a GC/MS method with EI for the separation and determination of chemical constituents in ether-extracted volatile oils of three traditional Chinese crude drugs, Jilin ginseng, *Radix Aucklandiae* and *Citrus tangerina* peels.⁹ Martens-Lobenhoffer *et al.* identified multiple components in a Chinese medicine pill used for the treatment of eye diseases.¹⁰ Liu *et al.* used HPLC and GC/MS for screening undeclared therapeutic substances in Chinese proprietary medicines.¹¹ Another

screening method using GC/MS for drugs and heavy metals in Chinese patent medicines was reported by Au *et al.*¹² Recently, chemometric techniques have been successfully used to process data from GC/MS analyses of formulated Chinese herbal drugs consisting of very complex mixtures derived from multiple medicinal plants.^{14–17} In these studies, a combined GC/MS and chemometric approach was used to analyze hundreds of essential constituents in various Chinese herbal medicines.

LC/MS has been playing a more and more significant role in Chinese medicine research because the technique is capable of characterizing active components ranging from small polar molecules to macromolecules such as peptides/protein, carbohydrates and nucleic acids. Recent scientific results and publications show that the application of LC/MS has been expanding rapidly into the area of structural elucidation and characterization of active components, in addition to valuable quantitative analysis. Several papers discussing LC/MS analysis of ginseng extracts and ginsenosides were published recently^{29–44} (see the next section for details). Lin *et al.* applied LC/ESI-MS to analyze chemical constituents of *Danggui* (rhizome of *Angelica sinensis*).⁴⁵ DiPaola *et al.* used LC/MS analysis to support the study of clinical and biological activity of an estrogenic herbal combination for the treatment of prostate cancer.⁴⁶ Xue and co-workers studied the precipitate produced in the preparation of a complex prescription of Chinese herbal medicines *Xiexin* (*Shanhuangxiexin Tang*) decoction by LC/MS/MS.^{47,48} The authors demonstrated that their LC/MS method was reliable for determining active components and their metabolites in support of pre-clinical and clinical studies. Kasimu *et al.* conducted a comparative study on the compositions and inhibitory activities of seven *Salvia* plants (*Danshen*) by examining their water and methanol extracts using LC/MS.⁴⁹ Liu *et al.* analyzed cholic, deoxycholic and chenodeoxycholic acids in the traditional Chinese medicine *Qingkailing* by LC/MS/MS.⁵⁰ After the identification of these acids, a quantitative method to determine cholic acid in the medicine was developed. LC/MS, combined with other analytical tools, was used by Hamasaki *et al.* to study highly selective antibacterial activity of novel quinolone alkaloids from a Chinese herbal medicine named *Wu-Chu-Yu*.⁵¹ Similarly, Gu *et al.* isolated and identified triptolide and triptidiolide and found that they were responsible for the anti-rheumatic properties of crude aqueous extracts of *Tripterygium wilfordii* Hook f (TWHF), which represented a novel class of immunosuppressive drugs with potential clinical utility.⁵²

Tandem mass spectrometry (MS/MS) is a powerful technique for detecting a target compound in complex matrices, including plant extracts^{53–56} and traditional Chinese medicines.^{57–60} The strength of this technique lies in the selectivity, high sensitivity and fast screening capabilities compared with many other separation and identification techniques. Ranasinghe *et al.* developed a rapid screening method using MS/MS for artemisinin and its congeners isolated from *Artemisia annua*.⁵⁶ Neutral loss MS/MS scans that are selective for different elimination reactions were used in order to screen for groups of related analogues present

in a hexane extract of *Artemisia annua*. In a review on the applications of LC/MS/MS in drug and biomedical analyses, Zhong *et al.* described the application of MS/MS techniques for the detection of nine corticosteroids illegally adulterated in traditional Chinese medicines.⁵⁷ LC/MS/MS was used by Wong *et al.* for analyzing proprietary Chinese medicines for the presence of toxic ingredients.⁵⁸ MS/MS has also been applied for structural characterization and identification of active components extracted from Chinese medicines.^{48,59,60} Wang *et al.* applied MS/MS for characterizing and determining active ingredients in the extracts from the decoction of *Xiexin*.⁴⁸ Collision-induced dissociation MS/MS was used by Xu *et al.* to differentiate ginsenosides Re and Rd, although the two ginsenosides have the same molecular mass.⁵⁹ Li *et al.* examined the anthraquinone derivatives from Chinese herbal medicine *Rhubarb* by using production LC/MS/MS scans.⁶⁰ Simultaneous determination of five ingredients in the sample extracts was demonstrated.

Capillary electrophoresis (CE)/MS is an attractive approach for the analysis of alkaloids in plant extracts and the success of CE/MS interface is mainly governed by the volatility of the CE running buffer.⁶¹ More volatile buffers such as formic acid, acetic acid, ammonium carbonate and ammonium acetate were found to be more compatible with CE/ESI-MS analysis. In a recent study, a non-aqueous buffer composed of ammonium acetate, THF and acetic acid was used to determine quinolizidine alkaloids.⁶² ESI-MS was used to study the transformation of diester-diterpene alkaloids in *Fuzi* during processing and concoction.

Among the various approaches to mass analysis, ion-trap MS has been widely reported to offer several advantages in terms of structural analysis particularly because of its MS/MS capabilities,^{37–42} while quadrupole mass filters demonstrate excellent sensitivity in quantifying active components in Chinese medicines.^{32–36,47–52} LC/MS with ion-trap analysis provides the capacity for multiple-stage tandem mass spectrometry (MSⁿ), a powerful tool for the unambiguous identification of unknown molecules. The ion trap can be used to fragment product ions further through multiple generations and hence is extremely useful for structure elucidation and identification of unknown components. Fuzzati *et al.* achieved the identification of 25 ginsenosides using reversed-phase LC separation and ion-trap MS analysis.³⁷ Tawab *et al.* reported ESI-MS analyses with consecutive fragmentation steps (MSⁿ) on an ion-trap mass spectrometer to identify the ginsenosides, to determine the sequence and linkages of the monosaccharides in both carbohydrate moieties in native bisdesmosidic ginsenosides and to elucidate the structure of products derived from enzymatic galactosylation of the natural products.⁴⁰ Fang *et al.* conducted MSⁿ experiments on an ion-trap mass spectrometer to investigate the profiles of steroidal saponin mixtures extracted from *Tribulus terrestris*, a traditional Chinese herb of long history used to strengthen the body's resistance, restore normal function of the body and promote blood circulation.⁴¹ Cui *et al.* used the ESI-MSⁿ technique to investigate the structures of three triterpenoid saponins in crude extracts from a typical Chinese herb *Acanthopanax senticosus* Harms.⁴² The MSⁿ spectra obtained were applied

to direct structure elucidation of the saponins extracted from crude plants. Employing the same ion-trap mass spectrometer, Cui *et al.* developed an LC/ESI-MSⁿ technique for the simultaneous determination of ginsenosides in plant extracts of ginseng.⁴³ Their paper demonstrated that ion-trap MS with both positive and negative ionization modes can be used to analyze ginsenosides rapidly in crude plant extracts and to provide structural information. In particular, fragmentation pathways of [M – H][–] ions resulted in several significant signals corresponding to the cleavage of the glycosidic bonds and sugars, allowed a straightforward structure elucidation from the MSⁿ spectra.

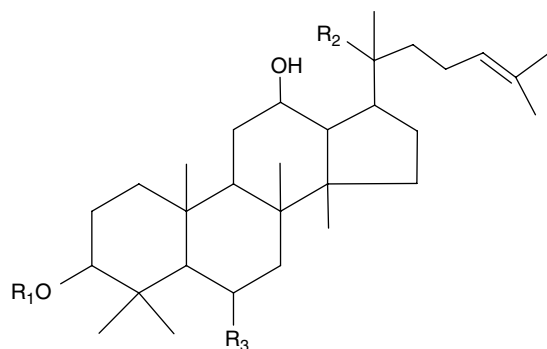
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) has been found to be an useful analytical tool for measuring polar components in Chinese medicines. Zhou *et al.* determined the molecular masses of eight ginsenosides and other components of total saponins in American ginsengs.²⁶ Another group determined the chemical compositions of polypeptides isolated from velvet antlers of sika deer and red deer that are used as drugs in traditional Chinese medicines.²⁷ With the support from TOFMS analysis, it was concluded that there are significant differences in the chemical properties and bioactivity of the velvet antler polypeptides from sika deer and red deer.

Mass spectrometry also plays a significant role in analysis of residual contaminants in Chinese medicines, an important safety issue in their use.^{11,61–74} MS has been used for the detection of steroids,^{61–63} pesticides^{64,65} and heavy metals,^{67–72} and also illegally added synthetic drugs in contaminated Chinese medicines.^{11,73–75} These applications provide very useful information for the purpose of quality assessment and standardization, a major issue that needs to be resolved before Chinese herbal medicines can be accepted by the international community.

GINSENG EXTRACTS AND GINSENOSES

Ginseng refers to plants of the genus *Panax*, which include *Panax ginseng*, *Panax quinquefolius* L. (American ginseng) and *Radix notoginseng* (*Sanqi*). *Panax ginseng* C. A. Meyer is one of the most popular natural tonics and is widely used in many Chinese medicinal prescriptions. Chemical and pharmacological studies on ginseng have demonstrated numerous constituents and actions. Active components in ginseng include O-glycosides of the triterpene dammarane structure, known as ginsenosides (ginseng saponins). The structures of many native ginsenosides have been identified (Fig. 1). Based on the difference in the aglycone moiety, ginsenosides are classified into three major groups: (20S)-protopanaxadiol (e.g. Rb₁, Rb₂, Rc, Rd, Rg₃, Rh₂), (20S)-protopanaxatriol (e.g. Re, Rf, Rg₁, Rg₂, Rh₁) and saponins derived from β-amyrin or a triglycoside of oleanolic acid (e.g. Ro).

GC/MS has been demonstrated to be a valuable approach for the analysis of volatile essential oils extracted from ginseng.^{76–79} Hou *et al.* reported a GC/MS method for separating and identifying several volatile oil components extracted from ginseng roots.⁹ Zhang *et al.* described an analytical procedure involving Soxhlet extraction of ginseng



Ginsenoside	R ₁	R ₂	R ₃	M.W.
Rb ₁	-Glc ² -Glc	-O-Glc ⁶ -Glc	-H	1108
Rb ₂	-Glc ² -Glc	-O-Glc ⁶ -Ara(p)	-H	1078
Rc	-Glc ² -Glc	-O-Glc ⁶ -Ara(f)	-H	1078
Rd	-Glc ² -Glc	-O-Glc	-H	946
Rg ₃	-Glc ² -Glc	-OH	-H	784
Rh ₂	-Glc	-OH	-H	622
Re	-H	-O-Glc	-O-Glc ² -Rha	946
Rf	-H	-OH	-O-Glc ² -Glc	800
Rg ₁	-H	-O-Glc	-O-Glc	800
Rg ₂	-H	-OH	-O-Glc ² -Rha	784
Rh ₁	-H	-OH	-O-Glc	638

Glc: glucose; Ma: Malonyl; Ara(p): arabinosein pyranose form; Ara(f): arabinosein furanose form

Figure 1. Structures of (20S)-protopanaxadiol (R₃ = H) and (20S)-protopanaxatriol (R₁ = H) ginsenosides.

rhizomes using diethyl ether followed by GC/MS analysis of the extracted oils.⁷⁹ GC/MS, however, is not amendable to the direct analysis of ginsenosides owing to their high hydrophilicity. Thus, ginsenosides were derivatized to yield the acetate, methyl ether or trimethylsilyl ether derivatives in order to increase their volatility prior to EI-MS and GC/MS analyses.^{1,80–83} After derivatization, EI-MS studies revealed useful information on the sequence of the sugar units in the oligosaccharide chain.⁸⁰ Ginsenosides extracted from ginseng were found to undergo alkaline cleavage by heating the ginsenoside component mixture with n-butanol.⁸¹ A satisfactory identification among isomers of (20S)-protopanaxadiol or (20S)-protopanaxatriol was obtained.⁸² A GC/MS method, combined with solid-phase extraction using a C₁₈ microcolumn, was developed and applied to analyze (20S)-protopanaxadiol or (20S)-protopanaxatriol in human urine after oral administration of ginseng.⁸³ Field desorption mass spectrometry has also been used for the direct analysis of ginseng saponins without derivatization.¹⁹

LC/MS with electrospray ionization (ESI) and collision-induced dissociation (CID) MS/MS has been used increasingly for the structure characterization of ginsenosides.^{29–44} Both positive and negative ionization of ginsenosides have been studied. Some alkali and transition metal cations form strongly bonded attachment ions with the ginsenosides.

As a result, their CID spectra of the metal attachment ions show a variety of structurally characteristic fragmentation patterns. Ackloo *et al.* conducted CID experiments on metal-attachment ions for the characterization of ginseng saponins.³⁴ Positive ion ESI-MS experiments with alkali metal cations such as Li⁺ and Na⁺ and transition metal cations such as Co²⁺, Ni²⁺ and Zn²⁺ were found to be useful in determining the molecular masses of ginsenosides, and their CID mass spectra show a variety of structure-related fragmentation patterns. The results can be used to determine the identity of the triterpene core, the type and attachment positions of sugars to the core and the nature of the O-glycosidic linkages in the appended disaccharides.

Van Breemen and Fitzloff claimed the first application of LC/MS to the analysis of ginsenoside standards and ginseng plant extracts.²⁹ Using positive ESI, they reported the successful use of the ginsenoside profile obtained from LC/ESI-MS analysis to distinguish one variety of ginseng from another. ESI-MS was also used by Mauri and Pietta to examine extracts of selected medicinal plants, including ginseng.³⁰ The technique allowed the identification of the main components of each extract, therefore providing typical fingerprints of the plants examined. Wang *et al.* reported the LC/MS/MS analysis of ginsenosides in *Panax ginseng* and *Panax Quinquifolius* by using an ionspray technique on a

triple-quadrupole mass spectrometer.³² The parent cations were further analyzed by MS/MS. The glycosidic linkages, the core and the attached sugar(s) of the ginsenosides can be determined from CID of $[M + H]^+$ ions. The authors quantified ginseng extracts by using Standard Reference Materials (SRMs) of ginsenosides and observed good linearity over a calibration range 2–2500 ng/ml⁻¹ for Rb₁, Rb₂, Rc, Rd, Re, Rf and Rg₁. MS/MS has also been applied to differentiate isomeric ginsenosides after LC separation, identify the terpene core and determine ginsenosides in plant extracts.³³

The quality and quantity of ginsenosites are different in various types of ginseng products. LC/MS profiles have been used to differentiate several ginsenosites in methanol extracts of various ginseng roots.^{35–36} Chan *et al.* developed a simple LC/MS method for the differentiation and authentication of specimens and commercial samples of *Panax ginseng* (Oriental ginseng) and *Panax quinquefolius* (American ginseng).³⁵ Another LC/ESI-MS method, recently published by Li *et al.*,³⁶ illustrates the use of a triple-quadrupole mass spectrometer for the analysis of ginseng plant extracts and for the differentiation of isobaric ginsenosides. The authors investigated the presence and concentration ratio of ginsenosides Rf and (24R)-pseudoginsenoside F₁₁ in Asian and North American ginsengs based on their baseline chromatographic separation and unambiguous identification using MS/MS. The great selectivity of the LC/MS/MS method is crucial for separating and distinguishing the two ginsenosides that have the same molecular formula. The LC/MS/MS technique was also highly sensitive and capable of quantifying ginsenosides down to the low-picogram range with better than 10% relative error. This is very helpful in clinical trials involving the evaluation or assessment of efficacy or safety of herbal medicines.

While LC coupled with quadrupole MS has been extensively used for the quantitation of ginsenosides, ion-trap and quadrupole time-of-flight (Q-TOF) MS provides several advantages in the structural analysis of ginsenosides and ginseng extracts. Several reports indicated that ion-trap MS with both positive and negative ionization modes can be used to analyze ginsenosides rapidly in crude plant extracts and to provide structural information.^{26,27,41–44} In particular, the fragmentation pathways of $[M - H]^-$ ions resulted in several significant signals corresponding to the cleavage of the glycosidic bonds and sugars, allowing a straightforward interpretation of the MSⁿ spectra for structure elucidation.⁴³ Recently, Cui *et al.* reported results of an investigation on the effect of metal (Li⁺, Na⁺, K⁺ and Ag⁺) cationization on CID for ginsenosides by using ESI ion-trap MS.⁴⁴ Metal-cationized ginsenoside molecules are found to have characteristic fragmentation pathways that are very useful for convenient screening of ginsenosides in mixtures.

Although rich structural information can be obtained through CID in the course of MS/MS or MSⁿ analysis, high-resolution MS (HRMS) analysis provides more detailed and accurate structural elucidation, especially for unknown degradation products or metabolites of ginsenosides.⁸⁴ Exact mass measurement for both parent and fragment ions from the HRMS analysis can provide information of the elemental

composition of an unknown. Figure 2 shows high-resolution ESI-MS/MS of ginsenoside Rc in the positive ion mode. The data were obtained from an analysis conducted on a Q-TOF mass spectrometer. The accurate mass measurement of fragment ions corresponds to specific structural parts of the ginsenoside within 10 ppm mass difference. Fragmentation pathways shown in the figure were obtained from the interpretation of the tandem mass spectrum and assignment of the accurate mass of each fragment ion. The results were found to be useful for the identification of metabolites of ginsenoside in support of *in vitro* metabolism and pharmacokinetic studies (detailed metabolism data are not shown). Preliminary results from structural elucidation indicate that the high-resolution MS/MS technique is extremely useful for supporting pharmacological, pharmacokinetic and metabolism studies of the active components from Chinese medicines.

DANSHEN (RADIX SALVIA MILTIORRHIZAE)

Danshen is dried roots of the medicinal plant *Salvia miltiorrhizae*. It is one of the most widely used and extensively studied herbs in the Orient, especially in China and Japan. The first recorded use of *Danshen* dates back more than 2000 years. *Danshen* is officially listed in the Chinese Pharmacopeia and used for the treatment of menstrual disorders, menorrhagia, insomnia, menostasis, blood circulation diseases and other cardiovascular diseases. Modern studies in recent years have confirmed many of its traditional properties and also uncovered some additional properties including anticoagulant and antibacterial activities and beneficial effects in patients with chronic renal failure.

Studies on the chemical composition of *Danshen* have been extensively reported in the literature. Components in *Danshen* can be grouped into two major classes: lipid soluble and water soluble. Table 1 lists compounds that have been identified in the literature. The lipid solubles, normally obtained by extraction with alcohol solvents, are rich in abietanoids and diterpene quinone pigments. More than 30 diterpenoid tanshinones have been isolated and identified from *Danshen*,^{85–91} and among them, the three representative bioactive components in the fraction are tanshinone I, tanshinone IIA and cryptotanshinone.

The major active ingredients in the water solubles include many plant phenolic acids which are mostly caffeic acid derivatives. The caffeic acid monomers include caffeic acid itself, danshensu, ferulic acid and the esters of caffeic acids. The dimers and trimers are the most abundant components and they include rosmarinic acid, protocatechualdehyde, protocatechuic acid, salvianolic acids, lithospermic acids, rosmarinic acid, etc. Apart from some benzoic acid derivatives, the majority of the polar phenolics in *Danshen* are caffeic acid derivatives.

In recent years, the water-soluble components of *Danshen* has attracted increasing attention because of their effectiveness in improving the renal function of rats with adenine-induced renal failure, as an antioxidant for the removal of free radicals and their potential in treating Alzheimer's disease.^{92–95} In a series of studies, Tanaka *et al.*⁹³ isolated

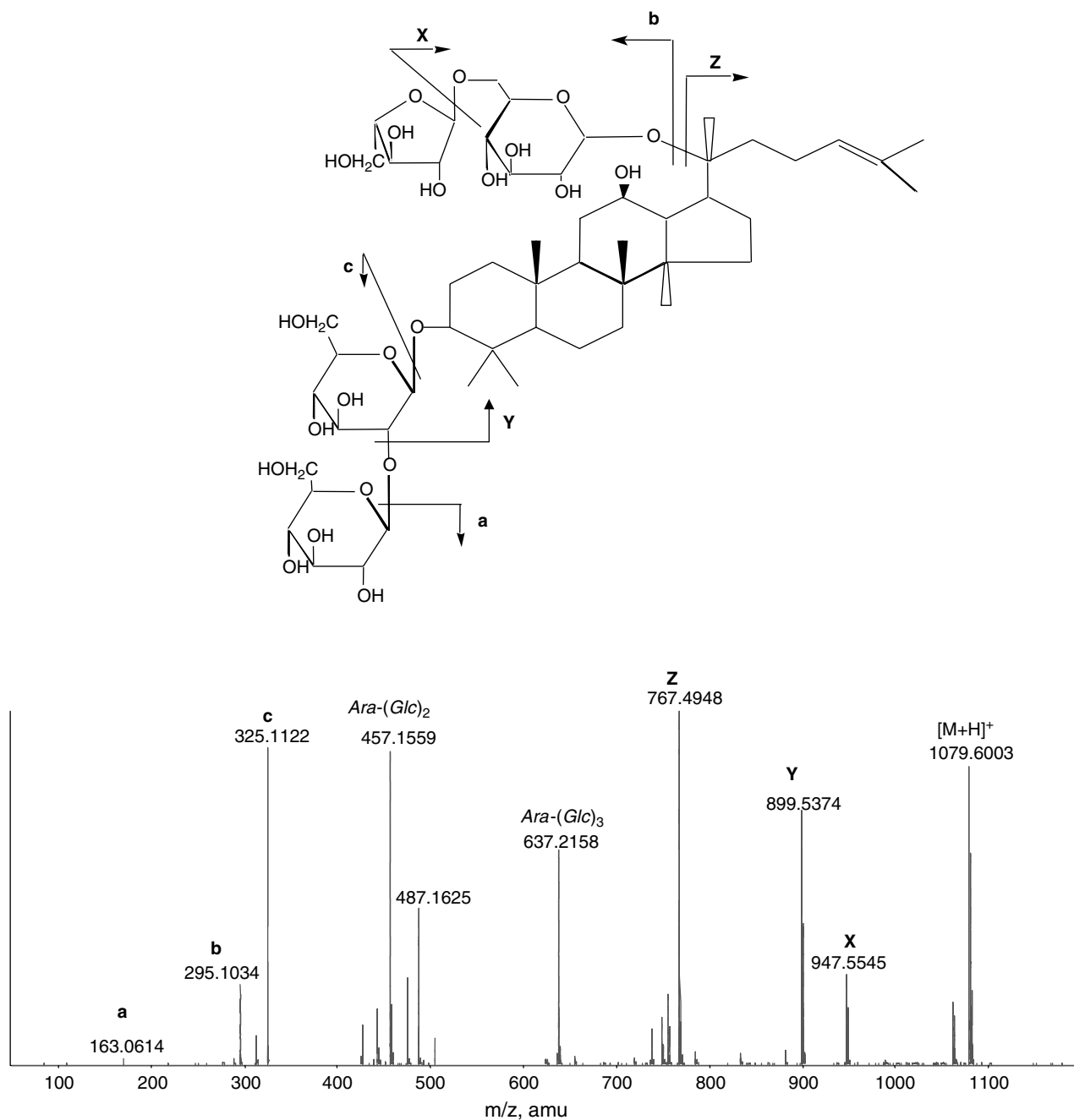


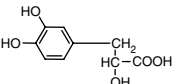
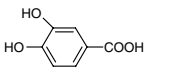
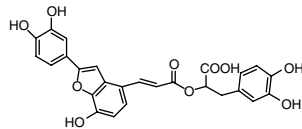
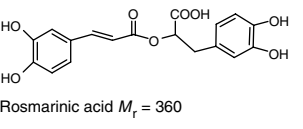
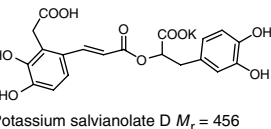
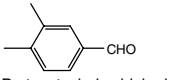
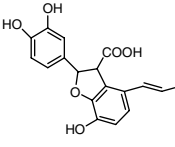
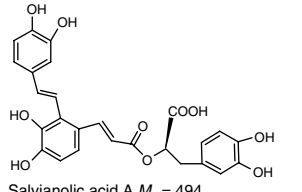
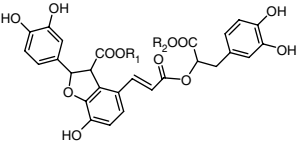
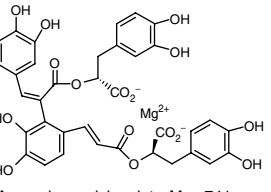
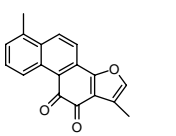
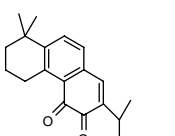
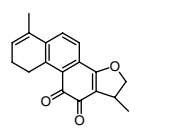
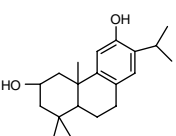
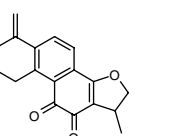
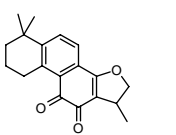
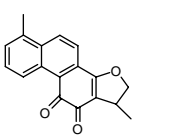
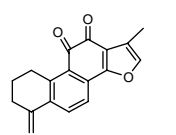
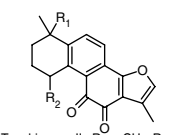
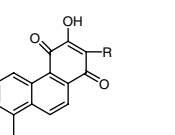
Figure 2. Accurate mass measurement of fragment ions and determination of fragmentation pathways of ginsenoside Rc obtained from positive ESI high-resolution MS/MS analysis.

the active components that exhibit an improving effect on renal functional parameters, namely marked reduction of glomerular filtration rate following adenine ingestion by injection of the components. The renal plasma flow and renal blood flow-rates were also increased in renal failure rats. One particular compound that shows this bioactivity was isolated by gel chromatography and then characterized by ^{13}C NMR and MS. Negative ion FAB-MS provided confirmation of the structure as the magnesium salt of lithospermate B. Zhou *et al.* used macropore resin separation to isolate rosmarinic acid, prolithospermic acid, lithospermic acid, magnesium lithospermate B, ammonium potassium lithospermate B and magnesium salvianolate E oligomers of caffeic acids.⁹⁶ Salvianolic acid A, salvianolic acid C and

potassium salvianolate D were also isolated in the *Danshen* injection fluid.

Currently, a comprehensive research program involving quality assurance and standardization of *Danshen* herb and its derived formulation products is on-going in our laboratories. A major part of this study involves systematic compositional analysis and comparison of active ingredients in *Danshen* originated from different locations and for different plant variations. The Chinese medicine made from natural products is subject to the influences of origin, age of growth, the harvest season and the processing method. These factors affect, to varying extents, the quality and efficacy of the Chinese medicinal products in terms of the amounts of bioactive ingredients. Toxic chemicals

Table 1. Structures of bioactive compounds in *Danshen*

Water-soluble compounds —				
 Danshensu $M_r = 198$	 Protocatechuic acid $M_r = 154$	 Salvianolic acid C $M_r = 492$	 Rosmarinic acid $M_r = 360$	 Potassium salvianolate D $M_r = 456$
 Protocatechuic aldehyde $M_r = 138$	 Pro-lithospermic acid $M_r = 358$	 Salvianolic acid A $M_r = 494$	 Lithospermic acid $R_1, R_2 = H$ $M_r = 538$ Lithospermate $R_1, R_2 = Mg^{2+}$ $M_r = 561$ Or $R_1, R_2 = NH_4^+ + K^+$ $M_r = 594$	 Magnesium salvianolate $M_r = 741$
Lipid-soluble compounds —				
 Tanshinone I $M_r = 276$	 Isotanshinone II _A $M_r = 282$	 Dihydrotanshinone I $M_r = 280$	 Salviol $M_r = 302$	 Methylenetanshinone $M_r = 280$
 Cryptotanshinone $M_r = 296$	 Dihydrotanshinone I $M_r = 278$	 Methylenetanshinguinone $M_r = 278$	 Tanshinone II _A $R_1 = CH_3, R_2 = H$ $M_r = 294$ Tanshinone II _B $R_1 = CH_2OH, R_2 = H$ $M_r = 310$ Methyltanshinonate $R_1 = COOCH_3, R_2 = H$ $M_r = 338$ Hydroxytanshinone II _A $R_1 = CH_3, R_2 = OH$ $M_r = 310$	 $R = -CH(CH_2)_2CH_2OH$ $M_r = 296$ $R = -CH(CH_3)_2$ $M_r = 280$ $R = -CH_3$ $M_r = 252$

such as pesticides, organic pollutants and heavy metals may also be present. Thus, conventional pharmaceutical Good Manufacturing Practice (GMP) production is difficult to apply directly to botanical products owing to the wide variations in the raw herb materials. This variability may occur from grower to grower and crop to crop and, furthermore, depends on harvest and post-harvest handling practices. Stability is more difficult to monitor and reference standards are more difficult to establish and obtain compared with synthetic drugs. It is because of these considerations that in Chinese herbal medicines, GAP (Good Agriculture Practice) guidelines aimed at the quality control of herbal plant production in the field has been promulgated recently by the Chinese government. The official GAP program for Chinese medicinal herbs started its trial period throughout China on 1 June 2002.^{97–98} Analytical techniques needed for the support of GAP studies of *Danshen* are also summarized in a book by the authors' research team.⁹⁹

Mass spectrometry and other analytical techniques have been used extensively for compositional and fingerprinting analyses to monitor the quality of *Danshen* from different sources, at different stages of growth and produced by different processing methods. Both LC/ESI-MS and TOFMS techniques have been employed. The tanshinones, which are polar and non-volatile compounds, can be easily identified by positive ion ESI-MS. The water solubles contain three major active ingredients, namely danshensu, protocatechuic

acid and protocatechuic aldehyde, which can be determined quantitatively by using the negative ion mode.^{99,100}

For routine fingerprinting analysis, direct sample introduction of *Danshen* sample extracts using the ESI-MS mode without LC separation has also been used. Here the relative abundances of major marker compounds in *Danshen* are used to confirm its presence, perform product QC or to differentiate the different *Danshen* varieties in the sample. Figure 3 illustrates the application of ESI-MS fingerprinting analysis for the QC of two popular OTC formulated drugs in China: an injection fluid and a pill product. Both drugs consist of complex formulations of different herbs but have water solubles of *Danshen* as the major active ingredients. Three major markers, i.e., Danshensu, protocatechuic aldehyde and rosmarinic acid (labiatic acid), are clearly evident in ESI-MS. The relative abundances of the marker components in the analysis were found to be fairly reproducible and readily quantifiable for process QC or product identification purposes.

The application of TOFMS for the analysis of *Danshen* extracts has also been carried out in our laboratories. In the last decade, we have witnessed a renaissance of the TOFMS technique, in particular in the biomedical area. This is due primarily to its well-known advantage of a wide mass range which is limited only by the ion detector. High ion transmission and the ability to detect ions of all masses at the same time provides sensitivity over the full spectrum equal to that in the selected-ion monitoring (SIM) regime. The first ESI-TOFMS instrument was constructed by Mirgorodskaya

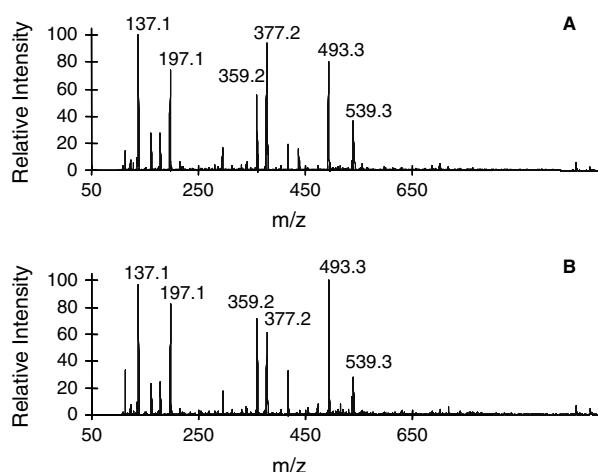


Figure 3. Negative ion ESI-MS fingerprinting analysis of *Danshen* injection fluid (A) and formulated *Danshen* pills (B) with water extract of *Danshen* as the major active ingredient. Peaks due to marker compounds include the following: 1, Danshensu, $C_9H_{10}O_5$, $[M - H]^-$ observed at m/z 197.1; 2, protocatechuic aldehyde, $C_7H_6O_3$, $[M - H]^-$ observed at m/z 137.1; 3, rosmarinic acid (labiatic acid), $C_{18}H_{16}O_8$, $[M - H]^-$ observed at m/z 359.2.

et al. in 1994,¹⁰¹ and a unit recently built in our laboratory has been used for routine analysis of *Danshen* extracts.

Typical ESI-TOFMS results from the analysis of the lipid-soluble extract of *Danshen* harvested in Spring 2002 at Zhongjiang, Sichuan, China are given in Table 2. Direct sample introduction by ESI resulted in abundant $[M + H]^+$ and $[M + Na]^+$ ions for nearly all *Danshen* samples, which helped in the positive identification of the compounds

of concern. With few exceptions, all components can be identified by the masses of their respective molecular ions with less than 10 mDa mass differences between observed and calculated values. Furthermore, the technique is being used routinely for screening and monitoring the quality of *Danshen* extracts. The high-resolution MS results are particularly useful in interpreting the complicated spectra obtained for complex mixtures such as Chinese medicines. The high precision (RSD 5–7%) and speed (<1 min per sample) of the technique offer high sample throughput, which is important in this type of studies involving large number of samples.

OTHER CHINESE HERBS

Ginkgo

Ginkgo (*Ginkgo biloba* L.) belongs to the *Ginkgo* phyla, which is one of the five main divisions of extant seed-bearing plants and contains only one living genus, *Ginkgo*. *Ginkgo* has been used for centuries as an invigorant medicine for warming the lungs, eliminating cough and sputum and facilitating recovery from hysteria. According to WHO reports, *Ginkgo biloba* leaf extract has been used for symptomatic treatment of mild to moderate dementia syndromes with symptoms such as memory deficiency, disturbance in concentration, dizziness and headache. In recent years, the extract of *Ginkgo biloba* L. has gained increasing importance in the health food and dietary supplement market globally because of the reported effects of *Ginkgo* extract on memory improvement and the prevention of cognitive decline.^{102–105}

Major bioactive compounds in *Ginkgo* include flavonoids (ginkgetin and isoginkgetin) and lactones (ginkgolides and bilobalides).^{106–107} Camponovo *et al.* used LC/thermospray

Table 2. ESI-TOFMS detection of active ingredients in the ethanol extract (lipid solubles) of *Danshen* harvested in Spring 2002 at Zhongjiang, Sichuan, China

Compound	Calculated mass		Detected mass		ΔM (mDa)	
	$[M + H]^+$	$[M + Na]^+$	$[M + H]^+$	$[M + Na]^+$	$[M + H]^+$	$[M + Na]^+$
Cryptotanshinone or isocryptotanshinone	297.1491	319.1310	297.150	319.123	1	–8
Tanshinone II _A or isotanshinone II _A	295.1334	317.1154	295.132	317.108	–1	–7
Tanshinone II _B or hydroxytanshinone II _A	311.1283	333.1103	311.121	333.109	–7	–1
Tanshinone I or isotanshinone I	277.0865	299.0684	277.085	299.072	–1	4
Methyl tanshinonate	339.1232	361.1052	339.120	361.111	–3	6
Miltirone	283.1698	305.1517	283.171	305.150	2	–2
Salviol	303.2324	325.2144	303.237	325.212	5	–2
Miltion	287.2011	309.1830	287.209	309.188	8	5
2-Isopropyl-8-methylphenanthrene-3,4-dione	265.1228	287.1048	265.102	287.092	–20	–12
Danshexinkun B	281.1178	303.0997	281.107	303.097	–10	–3
Danshexinkun C	253.0865	275.0684	253.082	275.060	–5	–8
Danshexinkun D	325.1440	347.1259	325.143	347.117	–1	–9
Tanshiniactone	269.1541	291.1361	269.154	291.121	0	–15

(TSP) MS to screen crude plant extracts and also different phytotherapeutic preparations containing *Ginkgo biloba*.¹⁰⁸ Since ginkgolides are easily ionized by TSP, on-line LC/TSP-MS is an efficient method for detecting a series of bilobalide and ginkgolides. The ionization was induced by the use of ammonium acetate and all mass spectra of the components show strong $[M + \text{NH}_4]^+$ peaks with little fragmentation. These pseudomolecular $[M + \text{NH}_4]^+$ ions allow qualitative and quantitative analyses of all ginkgolides in extracts with good precision, i.e. better than 10% RSD. Under optimum conditions, it was possible to analyze different phytotherapeutic preparations containing *Ginkgo biloba* with detection limits of 1 ng. Hodison used TLC, HPLC and GC/MS to identify and quantify free amino acids in *Ginkgo biloba*.¹⁰⁹ In the GC/MS analysis, amino acids were first converted to trifluoroacetyl-n-butyl esters to increase their volatility.

Safety issues of using *Ginkgo* have been raised recently. In 1996, the German Federal Institute for Drugs and Medical Devices extended the known adverse reactions associated with its use to include anaphylactic symptoms (shock, fever, leukocytosis and cardiac arrhythmia). It was found that some of allergen ginkgolic acids are toxic phenolic compounds. Many raw *Ginkgo* leaves contain only low levels of the active ingredients ginkgolides, bilobalides and flavone glycosides, but very high levels of ginkgolic acids. To facilitate the standardization of *Ginkgo* products, an authentic *Ginkgo* extract developed under standardized processing conditions is now available from the WHO. The availability of a standard extract greatly helps the standardization and calibration of chromatographic and mass spectrometric analyses. In order to detect *Ginkgo* acids at low levels, an LC/ESI-MS method has been developed.¹¹⁰ Three main phenolic acids in a chloroform fruit extract were isolated and detected by using LC/ESI-MS in the negative ion mode. The detection limits were in the range 0.1–0.25 ng.

Lingzhi

Lingzhi, *Ganoderma lucidum* (Fr.) Karst, along with its botanical relatives, is one of the best known medicinal herbs in the Orient. *G. lucidum*, a mushroom-like fungus, is used as a tonic drug. Fruit bodies and cultured mycelia are also prescribed in China to treat chronic hepatopathy, hypertension, bronchitis, arthritis and neurasthenia. Major compound classes in *Lingzhi* are triterpenoids, polysaccharides, nucleosides and nucleotides, alkaloids, steroids, enzymes, amino acids, polypeptides and trace metals. *Lingzhi* has attracted great attention because of its production of polysaccharides with anti-tumor and hypoglycemic activities. It also produces over 100 kinds of oxygenated triterpenes, many unique to *Lingzhi*, with various biological activities.^{111,112}

Lingzhi is the only known source of a group of triterpenes known as ganodermic acids, which have molecular structures similar to those of steroid hormones. Sye separated four unique ganodermic acids, A, B, C and D, and determined their molecular masses by MS.¹¹³ Ganodermic acids were identified as one of the major bioactive compounds in *Lingzhi*.¹¹⁴ Furthermore, *Lingzhi* was reported to contain the most active polysaccharides (long-chain sugars) among

medicinal plant sources, along with ergosterols, proteins, unsaturated fatty acids, vitamins and minerals. High hypoglycemic activity both in normal mice and alloxan-induced hyperglycemic mice has been induced by two polysaccharides–protein complexes, ganoderma B and C, obtained from hot water extraction. *G. lucidum* ganoderma B and C with hypoglycemic activity were fractionated on the basis of their hypoglycemic activity. The fraction obtained from ganoderma B was composed of 59.6% carbohydrate and 40.4% peptide and the fraction from C contained 81.5% carbohydrate and 18.5% peptide.^{115,116} A GC/MS analysis of the derivatized fractions showed the presence of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose and 2,3,4-trimethylglucose. The backbone and side-chains and degree of branching of ganoderma B and C were reported to correlate with water solubility and anti-tumor activities. Recently, Bao *et al.* used ESI-MS combined with NMR to characterize polysaccharides isolated from spores of *G. lucidum*.¹¹⁷ The techniques provide structural information and conformation and biological activities of the glucans.

Mahuang and aconitine

In recent years, MS has been used increasingly for the analysis of various alkaloids in plant extracts.^{62–63,118} *Mahuang* is a generic term applied to various types of Ephedra growing in China. *Mahuang* is the dried aerial parts of the plants, and has been used for the treatment of diaphoretic and antiasthmatic diseases.¹¹⁹ Ephedra's active ingredients include ephedrine, pseudoephedrine and norpseudoephedrine, which are strong central nerve stimulants. The major active constituent in Ephedra is the alkaloid ephedrine, which was isolated about 100 years ago and was structurally determined as α -(1-(methylaminoethyl)benzenemethanol.

Mahuang should be used under a physician's guidance because ephedrine is a stimulant that can cause severe reactions, from seizures to strokes and heart attacks. The US FDA reported over 300 cases of adverse reaction due to ephedrine in 1994 and 1995 alone. Classical methods for the determination of ephedrine in *Herba ephedrae* include the formation of the copper complex, TLC, ¹³CNMR and HPLC. CE in recent years has been increasingly used in the analysis of ephedrine and other polar alkaloids.¹²⁰ With little sample preparation, all six ephedrine alkaloids in commercial samples can be separated and analyzed. MS has been involved in the analysis of ephedrine in *Mahuang*.¹¹⁸ Betz *et al.* used GC/MS to separate and determine ephedrine-type alkaloids in dietary supplements containing *Mahuang*.⁸ MS and MS/MS have been proven to be necessary in the regulatory laboratory to assess the quality and safety of *Mahuang* and aconitine. Aconitine is an alkaloid that occurs in Chinese herbal medicines and must be used under strict regulation. It is a highly potent neurotoxin, which has been used as a source of poisons and medicines for centuries. Prompt preparation from certain aconitium roots can be used as medicines for analgesic, anti-rheumatic and neurological indications. However, untreated aconitium root is highly toxic and may cause paralysis, nausea and vomiting. Since this type of alkaloid on the one hand is toxic but on the other hand is an important active

ingredient for medical use, quantitative determination is important for safety considerations. CE coupled with ESI-MS was used to study the transformation of diester-diterpene alkaloids in *Fuzi* during processing and decoction.^{61,62} It was shown that aconitine, mesaconine, beiwutine, aconifine and hypaconitine were the main diester-diterpene aconitum alkaloids in *Fuzi*. The first four were hydrolyzed to benzoyleaconine and its analogues and hypaconitine was hydrolyzed to benzoylhypaconine after decoction. The relationship between structure and thermal stability of aconitum alkaloids was discussed. The reduction of all highly toxic alkaloids can be seen as evidence for the detoxification mechanism in *Fuzi*.

FINGERPRINTING STUDIES

The use of fingerprinting analysis for quality control and standardization of medicinal herbs has attracted intense interest in Chinese medicinal research in recent years, and several reviews have been published.^{121–123} In conventional drug analysis, fingerprinting is used to highlight the profiles of the sample matrix, which often is sufficient to give indications of the source and method of preparation. In herbal drugs, such a profile is dependent not only on the preparation processes but also the quality of the crude herb source material, which varies with different herb origins, sources, harvest times and pretreatment processes. The consistency and stability of the chemical constituents observed in the profiles thus reflect more than just the conditions of the drug preparation process, but also the source and quality of the raw herbs. The QA and QC of crude herbs by GAP guidelines as described earlier is therefore of prime importance to ensure the success of downstream GMP manufacturing. In both GAP and GMP, fingerprinting analysis is used to appraise the quality of the herbal material of concern, and the key is to develop links between marker compound-based chromatographic or spectroscopic profiles with the efficacy of herbal products.

TLC has been the most widely used classical method for fingerprinting analysis in Chinese medicines. Chromatographic profiles of major components are used to evaluate herbal growers and suppliers, to standardize raw materials and to control formulation and tablet content uniformity. Ideally, bioactive compounds or components should be identified. When this is not possible, important chemical marker compounds are developed to allow fingerprinting analysis for the assessment of batch-to-batch consistency. For example, in the analysis of valerian (*Valeriana officinalis*) and feverfew (*Tanacetum parthenium*), the two marker compounds are valerenic acid and acetoxyvalerenic acid in the former case and parthenolide and sesquiterpene lactones in the latter. In complex formulations, MS is often used to search for and identify these trace marker compounds. Lazarowych and Pekos illustrated how chromatographic fingerprinting and marker compound analysis could be used to make consistent herbal drug products.¹²¹

The fingerprinting chromatograms of *Danshen*, *Sanqi* and *Ginkgo* were studied by Sheng *et al.*¹²² Electro spray ionization

was used in HPLC/MS for the detailed profiling of active components. In fingerprinting analysis it is important to standardize all laboratory procedures to avoid artificial variations in results. The relative intensity of the peaks is important, and chromatographic fingerprints must be specific for the substance being analyzed. Hence it is necessary to check fingerprints obtained from related botanical products and known adulterants to ensure that the method developed can distinguish true from false identifications.

Kerns *et al.* discussed the use of LC/MS to obtain chemical fingerprints of botanical components in herbs for the identification of their derived products.¹²³ An analytical strategy based on LC/MS was developed and integrated into a high-throughput analytical program incorporating standard methods, template structure determination and structural libraries. The authors used LC/MS to characterize mixtures of taxanes from *Texus brevifolia* extracts and to develop a taxane database. The profiles generated provide specific fingerprints for chemical identification of the botanic material, while variation in the profiles are indicative of differences in sources, growing conditions and storage conditions. The sensitivity of the technique permits minimum sample preparation, thus saving analysis time and reducing unnecessary degradation of the components.

CONCLUSION

Over recent decades, a number of lead compounds and new natural products derived from medicinal herbs have been successfully isolated and identified, and great efforts have been made in chemical and pharmacological studies of Chinese herbs. Chemical analysis plays a central role in the development and modernization of Chinese medicines and mass spectrometry is rapidly emerging as the technique of choice in the identification of active ingredients, compositional analysis and fingerprinting studies. Up to now, the scientific basis of the majority of Chinese medicinal materials has remained poorly understood both chemically and pharmacologically. Concerted efforts and close collaboration between chemists and scientists in other disciplines will be needed to explore this exciting research area for the discovery of new remedies and novel lead compounds for new drugs of the future.

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