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Standardized Extracts of Chinese Medicinal Herbs: Case Study of Danshen (*Salvia miltiorrhiza* Bunge)

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ABSTRACT

The global trend of favoring healthcare with medicines or neutraceuticals derived from natural products has prompted an increasing interest recently on the development of herbal medicines. However, unlike synthetic chemical drugs, the pharmaceutical actions of herbal products often cannot be attributed to a particular compound, but owe rather to the synergy of a complex compound mixture. This calls for the urgent need to develop standardized extract to serve as reference materials for researches involved in drug discovery, product development and quality assessment. The emphasis of this review is to discuss the processes employed to prepare the extracts, and the techniques used to standardize their biochemical properties. Real cases, in particular those carried out in the author's own laboratory, will be used to exemplify major technical issues involved in these studies. Danshen (*Salvia miltiorrhiza* Bunge) in particular, will be used as a case study to illustrate the processes involved in extract preparation and standardization.

Key word: Chinese medicinal herbs, Danshen, standardized extract, reference materials

INTRODUCTION

Traditional Chinese Medicines (TCM) has been widely practiced for thousands of years in China and Eastern Asia. It is now an integrated part of the health care system in this part of the world; and currently in China, a strong push to modernize TCM through both scientific research and industrial development is underway[1-4]. As part of a global trend favoring healthcare with natural products, we are also witnessing in the past decades an increasing interest in Europe, US and other parts of the world on the use of natural herbs to formulate drug, dietary supplement or functional food products. In Germany, herbal medicines are categorized as medical products, and the estimate is that they account for about 10% of the market for prescription drugs, and nearly 30% of those of the OTC market. In the US, it is estimated that a quarter of the prescription drugs include at least one component which is derived from botanical plant extract or chemicals; and the market growth of herbal products such as St John's Wort, Ginkgo, Echinacea, Garlic, etc. has been phenomenon since the nineties.

In this article, we will review an important topic in TCM development, namely, the preparation and standardization of TCM extracts. The subject has attracted increasing attention recently because the key role played by TCM extract in drug discovery, health product development and laboratory research. Our emphasis here is place on the last aspect involving primarily discussions pertaining to the significance and application of standardized TCM extract in herb species authentication, product quality evaluation and the identification and quantification of bioactive ingredients. Real cases, in particular Danshen (*Salvia miltiorrhiza* Bunge), will be used to illustrate the significance of standardized extract and the techniques for their preparation.

HERBS AND HERBAL EXTRACT

In traditional TCM practices, the main forms of application for herbal drug are dried herb plants in the forms of slices, granules or powders. These are commonly called crude drugs, i.e., herbal plants which have had preliminary treatments according to traditional procedures such as cleaning, simple heating, baking or specialized cooking. The TCM drugs in real practice usually consist of a mixture of crude drugs in a complex herbal formulation or concoction. The concoction prescribed by the doctor is extracted and taken by the patient through oral administration. Another popular form of crude drugs is "Jingao", or "extract", which is the concentrated syrup form of the extract. In the 1999 New Drug regulations promulgated by Chinese SFDA, an active component(s) extracted from either a single herb or a concoction is classified as Class I new drug; whereas a group of active compounds in the whole extract or its chemical fractions is classified as Class II new drugs[5].

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In the 2005 Chinese Pharmacopoeia, 13 different kinds of "jingao" each prepared from a single herb have been recorded. A list of these extracts along with their major therapeutic functions and marker compounds are given in Table 1. In Europe and the US, extract is the main form of herbal products, and accounts for 95% of the TCM applications.

Over the last 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 on the chemical and pharmacological studies of Chinese herbs. However, up to now, the scientific basis of majority of the Chinese medicinal material remains poorly understood both chemically and pharmacologically. Compositional analysis of the herbal extracts is the key to unlock the secret of their effectiveness, and the typical way in such studies involves the preparation of herbal extracts, testing their pharmacological activity, isolating the individual components of the extracts, followed by instrumental analysis to identify and quantify the target active compounds of interest. Depending on the objective of the study, this multi-step process could lead to the preparation of three distinct types of intermediate extracts. The first is a "marker compound extract", in which a specified amount of a marker compound or a class of compounds with similar properties is included in the finished product. The marker compound is usually bioactive, although such activity may or may not represent the beneficial or therapeutic function of the entire extract. Examples of marker extracts include ginseng extract with ginsenosides as the markers, or licorice with flavornoids or glycyrrhizinic acids as the markers. The second type of extract is "active constituents extract" in which a specific component(s) with confirmed bioactivity has been enriched to a higher level than those present in the herbal plant itself. Examples of active constituents extracts include Gingko with enriched levels of glycosides or grape seed with enriched concentration of polyphenols. Table 2 lists some of the better known extracts of the above two types sold commercially as dietary supplements. The third type of extract is normally called an "active fraction", and is a term gaining increasing popularity in the TCM community in recent years. In these extracts, a broad base of compounds rather than a specific species or class of compounds are believed to be respon-

Table 1. Standardized extracts of Chinese medicinal herbs listed in Chinese pharmacopoeia (Ref 5)

Name of herb (pinyin)	Name of herb (English name)	Main therapeutic function	Marker species	% Range
Dahuang Liujingao	Extractum Rhei Liquidum	Purgative or laxative	Emodin and rhein	0.45
Gancao Jingao	Extractum Glycyrrhizae	Dispelling phlegm, relieving cough,	Glycyrrhizic acid	2.00
Yuanzhi liujingao	Extractum PolygalaeLiquidum	Expectorant	/	/
Lianqiao Tiquwu	Extractum Forsythiae Siccus	Heat-clearing and detoxifying	Phillyrin	0.50
Ciwujia Jingao	Extractum Acanthopanacis Senticosi	Strengthening the spleen and benefiting qi	Syringin	0.50
Jiang Liujingao	Extractum Zingiberis Liquidum	Strengthening the stomach and Qufeng	Extractum of ethyl ether	4.50
Yimucao Liujingao	Extractum Leonuri Liquidum	Promoting blood flow to regulate menstruation	Stachydrine hydrochloride	0.20
Huangqing Tiquwu	Extractum Scutellariae Siccus	heat-clearing and detoxifying	Baicalin	85.00
Dianqie Liujingao	Extractum Belladonnae Liquidum	Anticholinergic drug	Hyoscyamine	0.70-0.80

Table 2. Standardized herbal extracts sold as dietary supplement products in OTC market

Name of herb	Major therapeutic function	Marker species (wt% specified)	Ref
Ginseng G115®	Increase endurance and vitality, improving cognitive function	Ginsenosides (4%)	7
Devil's claw	Treatment of inflammatory disorders of the musculoskeletal system	Harpogosides (5%)	8
Feverfew	Reduces migraine frequency	Parthenolides (0.7%)	9
St Johnswort	An antidepressant	Hypericin (0.3%)	10
Gingko	Improve brain functioning, promote radical scavenging activity	24% flavoglycosides	11
Turmeric	Anti-oxidant, anti-platelet; anti-cancer	95% curcumin	12
Grape seed	Anti-oxidation, preventing atherosclerosis, anti-ulcer, prophylaxis of cataract	95% polyphenols	13

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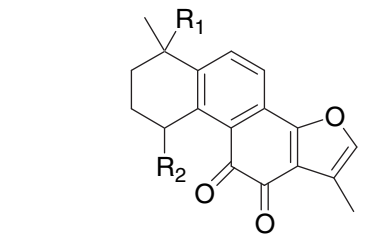
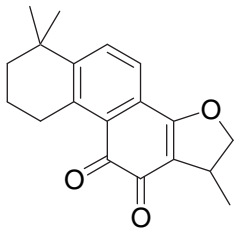
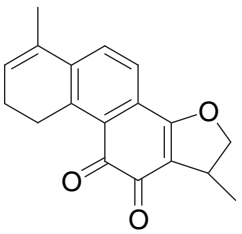


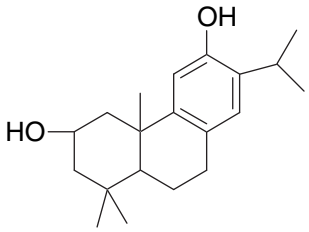
Tanshinone A $R_1 = CH_3, R_2 = H$
Tanshinone B $R_1 = CH_2OH, R_2 = H$
Methyltanshinonate $R_1 = COOCH_3, R_2 = H$
Hydroxytanshinone A $R_1 = CH_3, R_2 = OH$



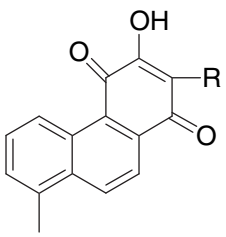
Cryptotanshinone



Dihydrotanshinone



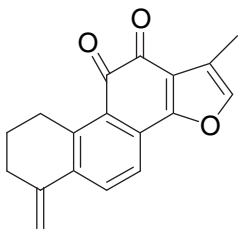
Salviol



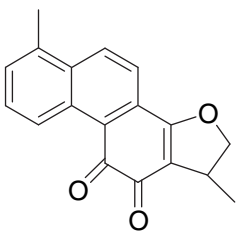
$R = -CH(CH_3)-CH_2OH$

$R = -CH(CH_3)-CH_3$

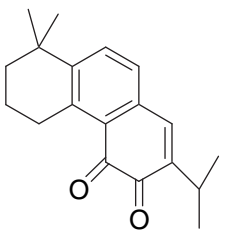
$R = CH_3$



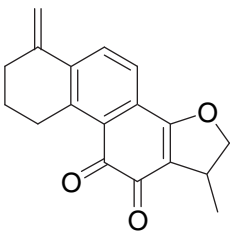
Methylenetanshinguinone



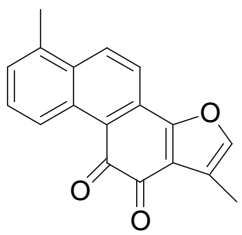
Dihydrotanshinone



Isotanshinone A



Methylenetanshinone

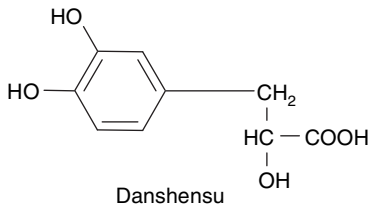


Tanshinone

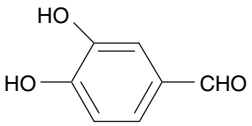
Figure 1. Major lipid soluble active components in Danshen.

sible for the bioactivity of the material. Many of the TCM extracts fall in this category, and will be the focus of our discussion in the text follows.

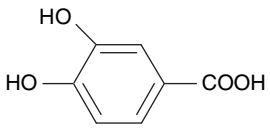
As a reference standard, the entire process for the preparation of extracts needs to be standardized in order to produce extracts with compositional uniformality, batch to batch consistency and consistent quality and property. Standardization requires the control of all variables which could potential affect the final quality of the extracts. These factors include the plant species,



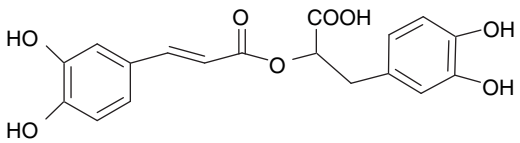
Danshensu



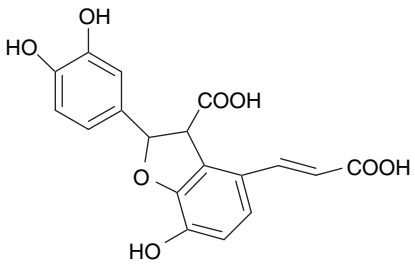
Protocatechuic aldehyde



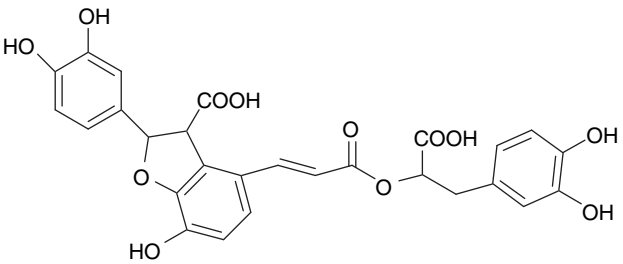
Protocatechuic acid



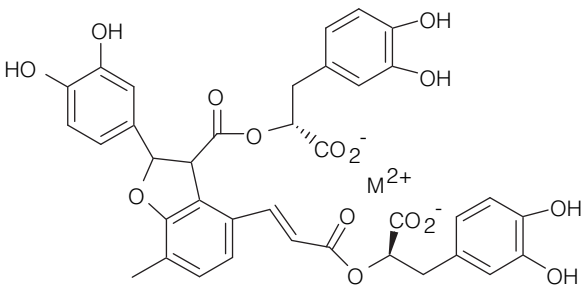
Rosmarinic acid



Pro-lithospermic acid

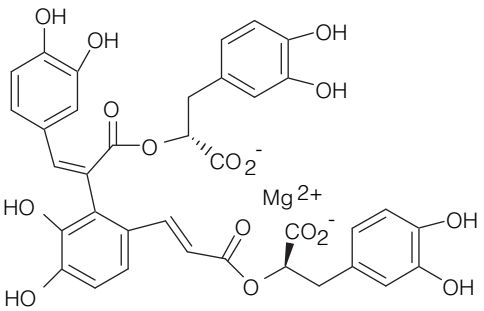


Lithospermic acid

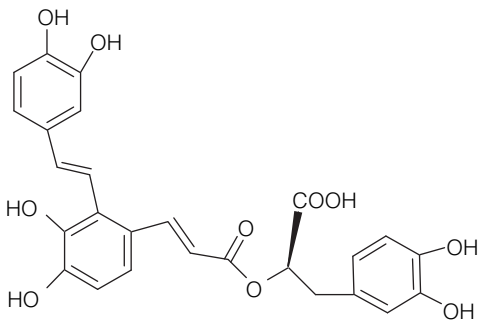


M^{2+}=Mg^{2+} Magnesium lithospermate

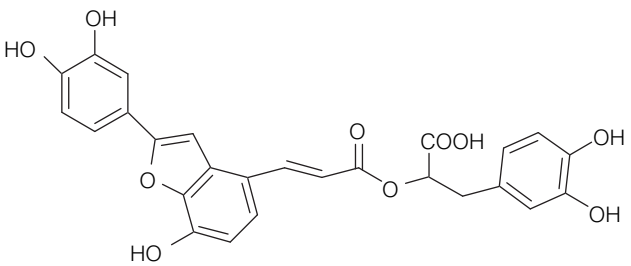
M^{2+}=NH_4^++K^+ Ammonium-potassium lithospermate



Magnesium salvianolate



Salvianolic acid A



Salvianolic acid C

Figure 2. Major water soluble compounds in Danshen.

the plant part, the primary processing step, post plant harvest, the extraction procedure and the manufacturing process leading to the products. A standardized extract should provide as detailed information as possible about the raw material as well as process conditions. Because of the complexity of the material, it is often difficult, and perhaps unnecessary, to establish an all-embracing chemical extract. Instead, several extracts from the same

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herb, each with its own composition and properties, can be produced to meet different objectives. Some common examples include the preparation of separate lipid soluble and water soluble extracts, or the separation of extracts based on compound types such as flavonoids, terpenoids, alkaloids, glucosides etc.

The extraction method should be selected and optimized based on two considerations: (1) it should contain at least the major and preferably all the active components in the original herb, and (2) it should reflect the efficacy of the original herb. The two objectives are met respectively by chemical analysis and bioassay in order to establish the chemical identity and to quantify the bioactivity of the active component. To better reflect the multi-components and multi-target nature of herb medicines, fingerprinting analysis coupled with computerized whole spectrum analysis has been developed recently to better represent the biochemical properties and compositional features of the material. As will be exemplified in later discussions, fingerprinting analysis of the extract provides a convenient means to establish the authenticity and assess the quality of the extract.

SIGNIFICANCE OF STANDARDIZED EXTRACT

The focus of our discussion is the development of standardized extract for the quality assessment of herbal drugs. To evaluate the quality of a drug means to establish the authenticity of the herbal species, and to determine the purity, consistency and potency of the material. The authenticity of a Chinese herb is usually established by reference to its descriptions given in the Chinese Pharmacopoeia, in which the sensory, macroscopical and microscopical characteristics of the herb is prescribed. Potency study refers to the determination of the intrinsic quality of the herb, i.e., the amounts and purity of the medicinal principles or active constituents present. Standardized extract plays a key role in both areas.

In TCM, it is a known fact that one herb may originate from more than one source and sometimes different plants are used under one common name. Even for the same species, the qualities of individual herbs vary greatly because of variation in geographical origin, cultivation practice, harvest time, and storage or processing conditions[1,14,30]. There are 540 different kinds of TCM listed in Chinese Pharmacopoeia (2005). Microscopic identification and chemical analysis are the two main techniques to distinguish different TCM species. To distinguish TCM through their apparent feature even under microscopy is often difficult. The technique is time consuming and strong expertise of the analyst is necessary for interpretation. Until recently, authentication by chemical analysis usually relies on comparative analysis using a single chemical compound as the marker or indicator species, e.g., the identification of American ginseng (*Panax quinquefolium. L*) using ginsenoside Rb1, Re and Rg1 as the marker compounds[15]. The method, however, has low specificity because these ginsenoside species are present in more than one herbal species besides American ginseng. Similar situation exists between Ligusticum chuanxiong and Radix Angelica Sinensis which share common ingredients of ligustilide and phthalide compounds[16]. The use of standard extract coupled with fingerprinting analysis (see sections follow) provides higher specificity and is more reliable.

Definable quality and quantifiable dose/bioactivity relationship are two major technical issues in the development of drugs or health products derived from natural herbs. It is now well documented that the pharmaceutical actions of herbal products often cannot be attributed to a particular compound, but owe rather to the synergy of a complex compound mixture. Currently, a common practice in natural product research is to select one or more compounds as either active or "markers" for quality assessment, similar to the situation described above in authentication exercise. Again, the application of bioactive extracts accompanied by reproducible fingerprints provide a more effective quality assessment system to truly represent its therapeutic effects.

STANDARD EXTRACT OF DANSHEN *(SALVIA MILTIORRHIZAE)*

I. *Properties of Danshen*

Danshen is the dried roots of the medicinal plant *Salvia miltiorrhizae*. It has been widely used to promote blood circulation, remove blood stasis, clear away heat, relieve vexation, nourish and cool the blood to relieve carbuncles[17]. Studies in recent years have confirmed many of its traditional properties and moreover, also uncovered some additional properties including its anticoagulant and antibacterial activities, and beneficial effects in patients with chronic renal failure[18,19]. Components in Danshen can be grouped into two major classes: the lipid soluble diterpene quinones and the water soluble phenolic acids. The lipid solubles, normally obtained by extraction with alcohol solvents, is rich in abietanoids and diterpene quinone pigments. More than 30 diterpenoid tanshinones have been isolated and identified from Danshen, and among them, the three representative bioactive components in the fraction are tamshinone I, tanshinone IIA and cryptotanshinone[20-24]. The contents of the tanshine quinones vary, ranging from 0.12% to 0.23% for tanshinones I, 0.02% to 0.32% for tanshinones IIA and 0.05% to 0.15% for methylene tanshinquinone. The major active ingredients in the water solubles include many plant phenolic acids which are mostly caffeic acid derivatives. The caffeic acids monomers include caffeic acid itself, danshensu, ferulic acid, and the ester forms of caffeic acids. The dimmers and trimers are the most abundant components and they include rosmarinic acid,

protocatechualdehyde, protocatechuic acid, salvianolic acids, lithospermsic acids, rosmaric acid, etc. Apart from some benzoic acid derivatives, majority of the polar phenolics in Danshen are caffeic acid derivatives.

In recent years, the water solubles of Danshen has attracted increasing attention because of its 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 disease[25-29]. Salvianloic acid consist of several classes, such as salvianloic acid A, salvianloic acid B, salvianloic acid C, etc. Salvianolic acid B is the most abundant member of salvianolic acids in Danshen[26-29], and has been assigned as the marker species for Danshen in the 2005 edition of Chinese Pharmacopoeia. The chemical structures of major water soluble species are shown in Figure 2. In our studies, salvianolic acids compounds in Danshen were selected as the marker components for the preparation of "standardized extract" of Danshen.

II. *Preparation of Danshen Extract*

The preparation of standardized herbal extract requires the careful control of the entire extraction procedure from herb selection, process optimization to product quality monitoring. Our lab is in the process of preparing a series of standardized herbal extracts intended for their use as reference materials for the authentication and quality evaluation of TCM products. The extraction protocol differs depending on the herb in question and the targeted active fractions or ingredients involved. As a case illustration, the process scheme for the preparation of water soluble active fractions from Danshen is illustrated in Figure 3.

The three-step process involves first the direct boiling of Danshen in water, followed by ethanol precipitation of the unwanted biopolymers, and finally back extraction with ethyl acetate to separate the active fractions containing polyphenols. The method is the suggested extraction method for the production of bioactive water soluble fraction of Danshen in the 2000 edition Chinese pharmacopoeia. The entire preparation procedure has been standardized, and the feed selection criteria, the extraction procedure, the product quality along with the quality control practice have been included in a SOP (Standard Operation Procedure) document.

The selection of genuine Danshen species with good quality is the first and most important step in the production

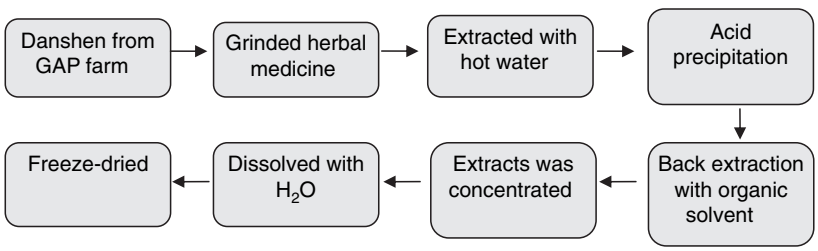

Figure 3. Schematics of process for the extraction of water soluble active fractions from Danshen.

Table 3. Compositional analysis of different batches of ZhongJiang Danshen showing the batch-to-batch consistency of the source herb

Danshen sample	Wt% Tanshinone II $_A$ (dry plant basis) (average of 3 runs)		Wt% Catealdehyde (dry plant basis) (average of 3 runs)	
	Quantified against chemical standard	Quantified against standard extract	Quantified against chemical standard	Quantified against standard extract
Batch 1	0.27	0.23	0.19	0.19
Batch 2	0.24	0.24	0.20	0.21
Batch 3	0.26	0.26	0.19	0.20
Batch 4	0.24	0.25	0.20	0.20
Batch 5	0.28	0.28	0.19	0.19
Batch 6	0.21	0.20	0.21	0.21
Batch 7	0.23	0.21	0.17	0.18
Batch 8	0.29	0.30	0.17	0.17
Batch 9	0.34	0.34	0.24	0.25
Batch 10	0.23	0.23	0.14	0.15
Ave	0.26	0.26	0.19	0.21

*Student t test gives $< t_{0.05} = 2.26$ at $P > 0.05$, showing the absence of bias between two sets of data.

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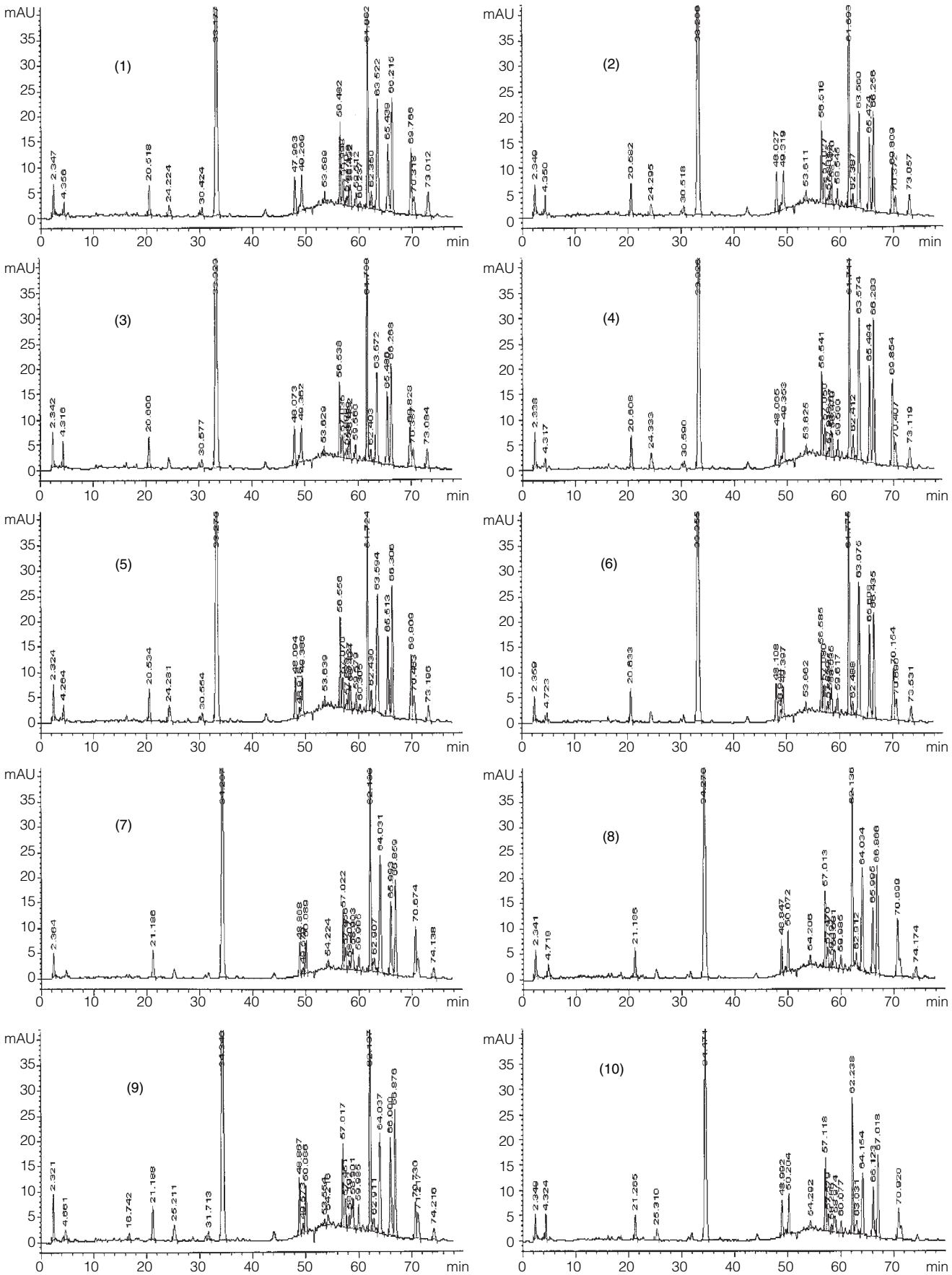

Figure 4. HPLC chromatograms showing the compositional consistency of different batches of Danshen samples collected from GAP farms in ZhongJiang, SiChuan, China.

Table 4. Concentrations of salvianolic acids in Danshen samples of different plant species or geographic locations

Plant species	Origination (province in China)	Wt % yield (based on dry weight of Danshen plant*)							
		I	II	III	IV	V	VI	VII	Total
Salvia miltiorrhiza	Sichuan GAP farms	0.057	0.028	--	0.069	1.94	--	3.17	5.52
Salvia przewalskii Maxim	Yunnan, cultivated farms	0.088	0.570	0.068	--	2.40	--	2.46	5.58
Nan Dansheng	Jiang Xi, cultivated farms	0.020	0.068	--	0.056	0.36	0.053	7.04	7.60
Salria sinica	An Hua, cultivated farms	0.046	0.370	--	--	1.41	--	6.00	7.83

*I. protocatechualdehyde, II. caffeic acid, III. methylrosmarinate, IV. salvianolic acid A, V. rosmarinic acid, VI. salvianolic acid C, and VII. salvianolic acid B.

of standard extract. The Danshen plant we studied were collected from certified GAP (Good Agriculture Practice) farms[30]. The GAP program implements comprehensive quality guidelines in seed selection, species authentication, environmental control, cultivation practice and post-harvest processing. These processes ensure the production of genuine Danshen extract which is contamination-free, with good quality, and in controllable yield.

The batch to batch consistency of the Danshen extract obtained from the standardized extraction process given in Figure 3 can be seen in Figure 4 and Table 3. Figure 4 shows the HPLC chromatograms of Danshen extracts obtained from different batches of raw Danshen plant collected in the GAP farm. The similarity of the composition among these samples, or the reproducibility of the extraction process, is visually evident. The similarity of these chro-

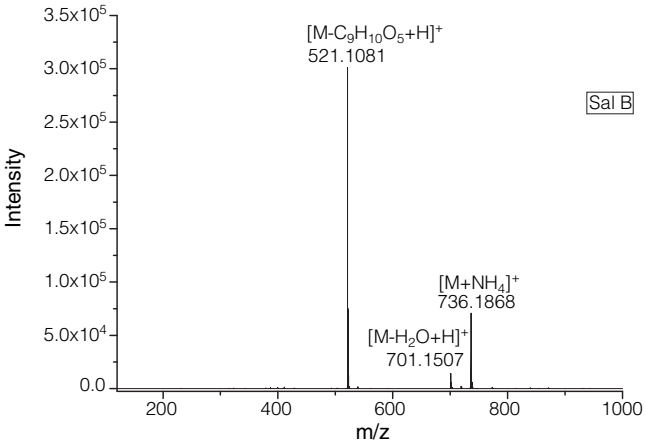

Figure 5. Mass spectra of salvianolic acid B in "standardized extract" of Danshen by HPLC-ESI-TOF/MS.

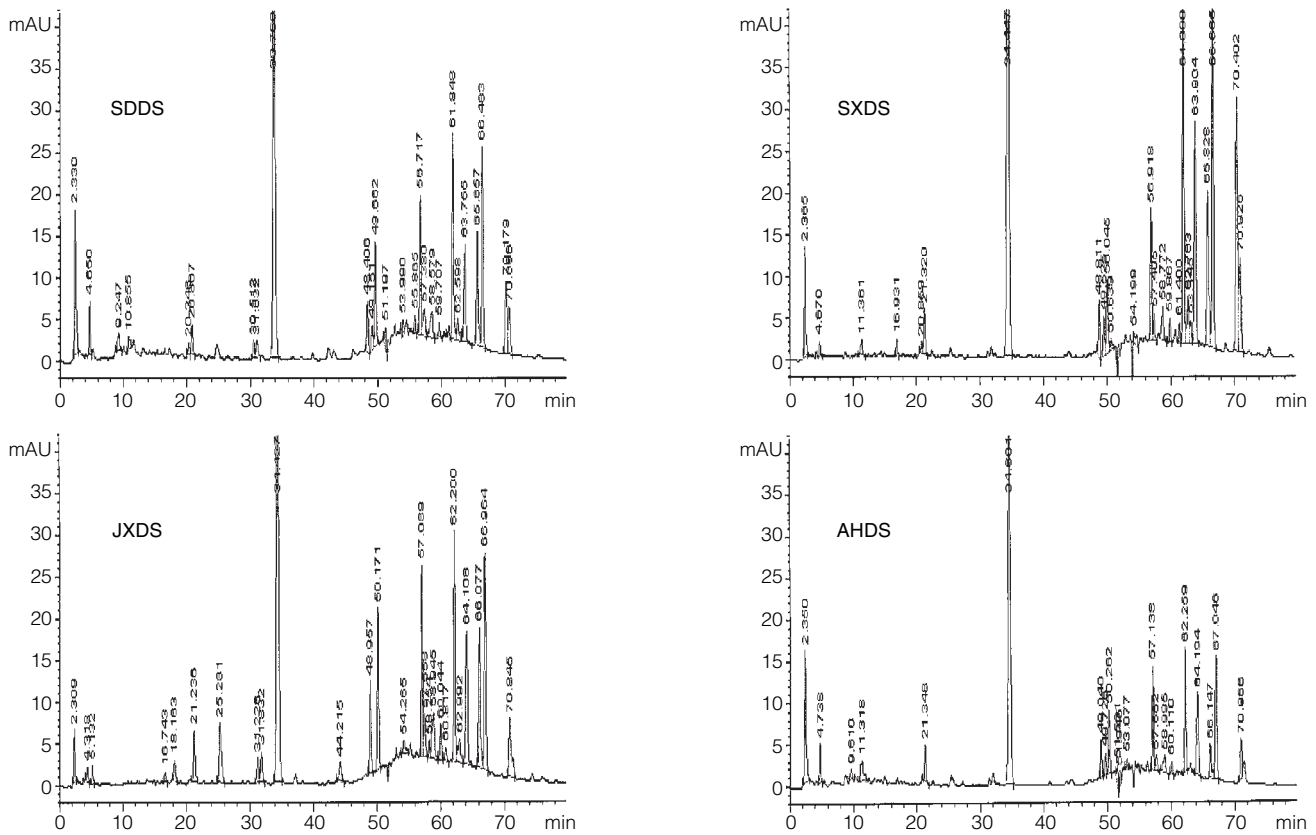

Figure 6. HPLC chromatograms showing the difference in composition among Danshen samples originated from different geographical locations. (SDDS: SanDong; SXDS: SanXi; JXDS: JiangXi; AHDS: AnHuie).

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matograms or fingerprints can actually be quantified using chemometric techniques as will be described later in the next section. The excellent agreement among these samples is demonstrated by the high similarity index calculated (0.97; with a perfect match of 1.0). In the chromatograms, the most intense peak observed, with a retention time of 41.0 min, is Salvianolic Acid B, the intended index species of the extract. Figure 5 shows that positive confirmation of the compound by HPLC-TOF-MS analysis. The reproducibility of the extracts is also evident from the data given in Table 3, in which the yields of liposoluble thanshinone IIA and water soluble protocatechusic aldehyde in the extracts of the 10 different Danshen batches are compared. A batch to batch comparison shows that the yields of the two compounds agree within 15-20%. Two different calibration systems were used for yield determinations: (1) standard reference compounds of tanshinone IIA and Protocatechausic aldehyde and (2) standard Danshen extract prepared in the study. The two sets of yields obtained are in excellent agreement, indicating the validity of using the standardized extract as reference materials for the quantification of active ingredients and quality assessment.

One of the major applications of the extract is to use it as a reference material to discriminate Danshen samples of different origin, different grade or different plant species. In Figure 6, the HPLC fingerprint (see next section for detailed discussions) of ZhongJiang Danshen is compared with those of other Danshen species originated from different geographic locations. The large variations in composition among these samples are clearly visible. In Figure 7, the retention times and the intensities of all the peaks are normalized against the most intense peak in the chromatogram. Each of the normalized peaks are then converted to "bins" with equal width, with its peak height directly proportional to the peak area for the ease of visual examination. Reference against the bar coded HPLC fingerprints of the standard extract, it is clearly evident that the compositions of the four Danshen samples originated from different locations are quite different. The large compositional variations among different Danshen samples can also be seen in Table 4, in which the concentrations of salvianolic acids in different Danshen samples are listed.

As expected, the extraction conditions affect greatly the composition of the extract. Tables 5 and 6 illustrate respectively the differences in yields of tanshinones and Salvianolic acid among extracts obtained from the same Danshe herb but using different extraction solvents and extraction techniques. The results re-emphasize the importance of standardization and quality control in the extraction process. As a reference standard, the storage

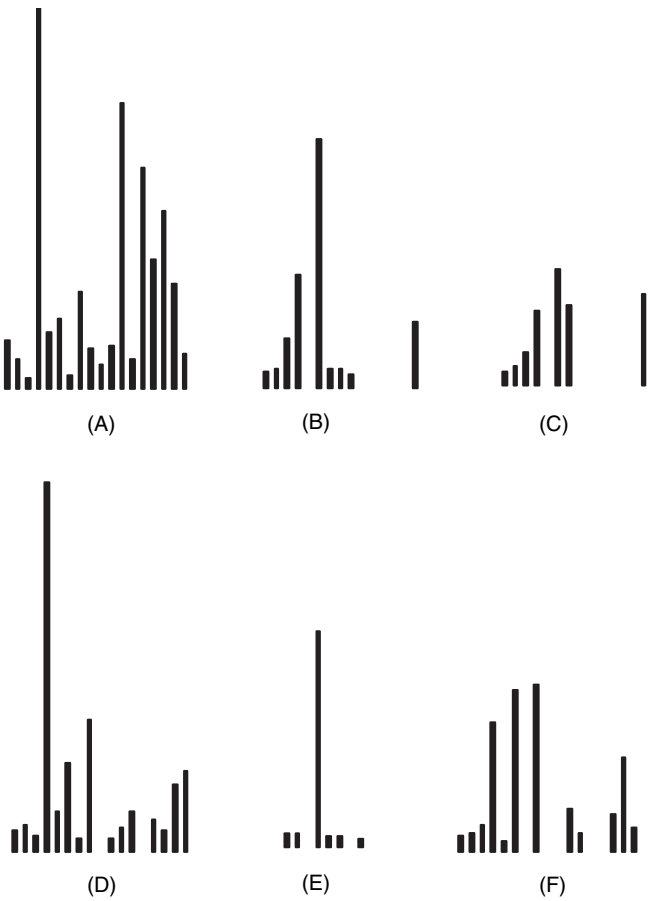

Figure 7. Bar coded HPLC fingerprints of extract of Danshen originated from different geographic locations. (A) IVDS; (B) GXSC; (C) XJSC; (4) NIDS; (E) NDS; (F) TDS. HPLC fingerprinting barcode of components from *Salvia miltiorrhiza* Bge. and other Salvias. (A) IV *Salvia miltiorrhiza* Bge.; (B) Salvia przewalskii; (C) Salvia deserta; (D) Salvia paramiltiorrhiza; (E) Salvia bowleyana; (F) Salvia yunnanensis.

Table 5. Yield of salvianolic acid B from Danshen extracted by different methods

Extraction solvent and conditions	% Yield of salvianolic acid B
(1) Water extraction, HCl acidification, back extracted with acetyl acetate	3.3
(2) Water extraction, add NH4OH, neutralized by HCl, back extracted with acetyl acetate	3.0
(3) Ethanol extraction, add NH4OH, neutralizied by HCl, back extracted with acetyl acetate	2.9
(4) Same as (1) but under ultrasound Assisted extraction	3.8
(5) Same as (4) but acidify with acetic acid rather than HCl	2.9
(6) Same as (2) but with ultrasound assisted extraction	2.5
(7) Same as (3) but with HCl replaced by acetic acid	1.0

stability is also an important property to check. The extract was stored in dry box at room temperature for a time period of 18 months. Aliquots were taken periodically to check the stability of the extract. The parameters checked include the physical properties such as color, smell and taste, the yields of the marker compounds, the bacteria contents and the reproducibility of the fingerprints. Both TLC and HPLC were used to assay the composition of the samples. The results showed that there was no detectable change after 18 months storage.

III. *New Extraction Technology*

The traditional method to extract active ingredients in TCM is to boil the material in water, or leaching it with water or ethanol. To isolate and enrich the bioactive molecules, which are usually smaller molecules of submetabolites, the aqueoud extract is then treated with ethanol to precipitate the inert biopolymers such as lignans, polysaccharides, polypeptides, proteins and carbohydrates, etc. In recent years, a diverse of new technologies have been developed for the extraction and separation of bioactive components in TCMs[4,31-33]. Table 7 lists some examples to illustrate how these techniques have been successfully applied to licorice and Danshen, the two herbs which have been studied more extensively in the authors' own laboratory in recent years.

FINGERPRINTING TECHNOLOGY

The development of different compositional fingerprinting techniques aiming at the authentication, quality control and standardization of raw herbs and their derived products has attracted intense interest recently[57-66]. In conventional drug analysis involving synthetic chemicals, the determination of a single or few active ingredients or marker species is usually sufficient for product identifica-

Table 6. Yields of tanshinones from Danshen extracted by different methods (Ave of three runs)

Extraction method	Cryptotanshinone extracted (μg/g)	Tanshinone I extracted (μg/g)	Tanshinone IIA extracted (μg/g)
Boiling water reflux (method listed in Chinese pharmacopeia)	163 ± 27	88 ± 14	1151 ± 60
Leaching in hot ethanol (50°C)	148 ± 17	66.0 ± 5.5	1057 ± 103
Leaching in cold ethanol	185 ± 22	88.4 ± 10.0	1490 ± 206
Ultrasonic extraction with ethanol	324 ± 37	151 ± 22	2334 ± 105

Table 7. New extraction techniques illustrated by case studies of Danshen and Licorice

Extraction or separation techniques	Targeted active component(s) or fraction (s)	Ref
Ultrasound assisted extraction	glycyrrhizic acid from licorice root	34, 35
Ultrasound assisted extraction	Compare with static extraction, alcohol solvents for the extraction efficiency of licorice using Danshensu and Protocadechualdehyde as markers	36
Microwave assisted extraction	Glycyrrhizic acid from licorice root	37, 38, 39
Microwave assisted extraction	Comparison between reflux extraction and moicrowave extraction for glycyrrhizic acid from licorice root	40
High speed counter-current chromatography	Major active constituents in Salvia miltiorrhiza Bunge	41
High speed sounter-surrent shromatography	Tanshinones from *Salvia miltiorrhiza* Bunge	42
High speed counter-current chromatography	Separation of diterpenoids from *Salvia miltiorrhiza* b	43
Multidimensional high speed counter-current chromatography	Tanshinones from *Salvia miltiorrhiza* Bunge	44
High speed counter-current chromatography	Glycyrrhizic acid from licorice	45
High speed counter-current chromatography	Inflacoumarin A and licochalcone A from licorice	46
Macro porous absorption resin	Enrichment of water solubles in Danshen	47, 48
Macro porous absorption resin	Glycyrrhizic acid and KanCaiGan from licorice root	49, 50, 51
Macro porous absorption resin	Isolation of salvianolic acid B from Danshen	52
Accelerated solvent extraction	General applications	53
Aqueous two phase extraction	Separation and purification of glycyrrhizic acid from licorice	54, 55, 56

tion or quality assessment. For herbal products with their complex and sometimes ill-defined active components, fingerprinting analysis is a better approach to appraise the quality of the herbal material of concern. In year 2000, SFDA of China officially published the requirement to use fingerprinting techniques for the authentication and quality appraisal of TCH derived injection fluids[57]. Similar suggestions can also be found in the documents published by WHO[67], USFDA[68], EMEA[69], German Commission E[70], British Herbal Medicine Association[71] and Indian Drug Manufacturer's Association[72]. A recent publication by USFDA also stated that fingerprinting technique is acceptable for botanical drug products and botanical drug substance when submit CMC (Chemical Manufacture and Control) information for the application of Investigational New Drugs(IND).

The two major types of fingerprinting techniques are chromatography and spectroscopy. Instrumental analysis based on chromatographic techniques of HPLC (High Performance Liquid Chromatography), HPTLC (High Performance Thin Layer Chromatography), CE (Capillary Electrophoresis); and spectroscopic techniques of FTIR (Fourier Transform Infrared), NIR (Near Infrared), and NMR (Nuclear Magnetic Resonance); or different versions of MS (mass spectrometric) techniques such as ESI-MS (Electrospray Ionization MS), TOF-MS (Time-of-Flight MS) have all been successfully applied to the fingerprinting of a wide variety of TCMs or their derived products. In Table 8, a list of different instrumental techniques which have been applied to the fingerprinting analysis of Danshen is given. The list is not meant to be exhaustive but to exemplify some of the applications which have been reported recently in the literature. In Table 9, some recent examples on the application of different chemometric techniques in TCM fingerprinting is shown.

I. *Chromatographic Fingerprinting and Similarity Calculation*

HPLC is the technique which has been used most extensively for the fingerprinting analysis of TCM. In herb authentication, the chromatographic fingerprint is matched against those of the reference standard. Due to the complex fingerprints of the herbal samples and operational variations in chromatographic analysis, an accurate analysis and interpretation of the chromatogram in a typical chemical fingerprinting analysis poses a great challenge to analysts The simplest method to compare complex fingerprint is by visual comparison. The technique is simple to practice, and could be quite effective in a lot cases. In the bar coded HPLC chromatograms given earlier in Figures 7 and 8, it is clearly seen that the fingerprints can be effectively used to discriminate different Danshen species or confirm the same ones. In many cases, however, visual judgment can be subjective

Table 8. Different techniques applied to the fingerprinting analysis of Danshen

Technique applied	Fingerprinting objectives	Ref
High performance liquid chromatography-diode array detector	Species authentication and quality assessment in good agriculture practice study	73
High performance liquid chromatography coupled with liquid chromatography-mass spectrometry-mass spectrometry	Quality assessment and structural identification of bioactive components	74, 75
High performance liquid chromatography-electrospray mass spectrometry	Quality assessment and compound identification in good agriculture practice study	76
High performance liquid chromatography-atmospheric pressure mass spectrometry	Quality assessment and compound identification in lipid soluble fraction	77
Time of flight-mass spectrometry	Rapid fingerprinting of tanshinones for species identification	78
High resolution mass spectrometry	Overview of mass spectrometry fingerprinting in TCMs	79
High performance capillary zone electrophoresis	Fingerprinting with Danshen Su and protocadechualdehyde as markers	80
High performance capillary zone electrophoresis — micellar electrophoretic kinetic Chromatography	Fingerprinting based on tanshinones in lipid soluble fractions	81
High performance counter current chromatography	Quantification of salvianolic acid B	82
High performance thin layer chromatography	Fingerprinting of lipid and water soluble fractions for species authentication and quality assessment	83
Infrared spectroscopy	Species authentication and discrimination	84
Near infrared spectroscopy	Quality assessment and authentication of raw herb and herbal extracts	85, 86

Table 9. Chemometric technique used in fingerprint analysis

	1	2	3	4
Similarity evaluation	Quality control of Pseudostellaria heterophylla (Miq.) Pax[92]	Critical value determination on similarity[100]	New approach on similarity analysis[103]	Distinguishing Chinese Angelica from related umbelliferae herbs[105]
Principal component analysis	Quality control of Qingkailing injection[93]	Near-infrared spectroscopy for classification of licorice[96]	Classification of olive oils[97]	Quality assessment of TCM[104]
Hierarchical clustering analysis	Quality control of Panax quinquefolium. L [94]	GC–MS fingerprint of *Pogostemon cablin*[101]	Fingerprint analysis of Flos Lonicerae japonicae[106]	Fast olive oil fingerprinting of TOF/MS[107]
Partial least square	Correlation of PTR-MS spectral fingerprints[99]	Data pretreatment methods for handling of complex data[102]		
Artificial neural network	Prediction of cultivars and vintage[95]			
Soft independent modeling of class analogies	Classification[98]			

and non-quantitative, and computerized chromatographic matching becomes necessary.

Owing to recent advance in chemometrics techniques, more quantitative methods such as similarity analysis and clustering analysis based on the entire chromatographic fingerprint are now becoming available. Before fingerprinting analysis, the raw chromatograms of concern have to be standardized. The standardization process includes the selection of "common peaks" in the chromatogram, and the normalization of the retention times and peak areas of all the common peaks against the peak of a marker species. The marker species is either the most prominent peak in the chromatogram or a peak with known bioactivities. In HPLC fingerprints, the similarity analysis is usually performed by a professional software named Similarity Evaluation System for Chromatographic Fingerprint, which was recommended by SFDA (State Food and Drug Administration) of China[87]. The software quantifies the similarity indexes among different chromatograms by calculating the correlative coefficient and/or cosine value of the vectorial angle.

Using the above method, for instance, the similarity indices quantified by similarity calculations among the chromatograms of Danshen presented in Figure 4 are all above 0.98 (with 1 being the perfect match), indicating the high reproducibility of the compositions of the extract. Some other examples including similarity matching followed by various cluster analysis techniques can be found in the references given in Table 9.

II. *Spectroscopic Fingerprinting and Cluster Analysis*

The second type of fingerprinting techniques involves various spectroscopic methods of which the more popular ones include FTIR, NMR and NIR. Mass Spectrometric analysis is usually coupled with chromatography in hyphenated systems such as LC-MS or GC-MS, for which many case studies can be found in the references given in Tables 8 and 9. One example of fingerprinting Danshen extract by direct TOFMS analysis is given in Figure 9, which shows the well resolved mass peaks of the three thansinones in the lipid soluble extract of Danshen[78]. The most attractive feature of the technique is its fast analytical speed, in the order of seconds per sample. The technique is thus ideally suited for situations requiring high throughput analysis.

Similar to chromatographic analysis, various chemometric techniques have also been applied to analyze the spectral data and among them, the more popular ones include Principle Component analysis (PCA), Hierarchical Cluster Analysis (HCA), K-Nearest Neighbor Analysis (KNN) and Partial Least Squares (PLS). Information on the principles and applications of these techniques can be found in many good reviews[88-90].

One of the spectroscopic techniques studied more extensively in our laboratory for TCM fingerprinting is Near Infrared (NIR). Results illustrated in Figure 10 (A) and (B) shows the capability of NIR in discriminating two different plant species of Danshen -- S.miltiorrhiza Bge and S.paramiltiorrhiza[91]. Figure 10 (A) compares the two original NIR spectra, which are very difficult to distinguishable visually. PCA was performed to the whole spectra to examine the qualitative difference of the spectra in the principal component space, with each principle component explains independent variations within the sample. The first two principle components

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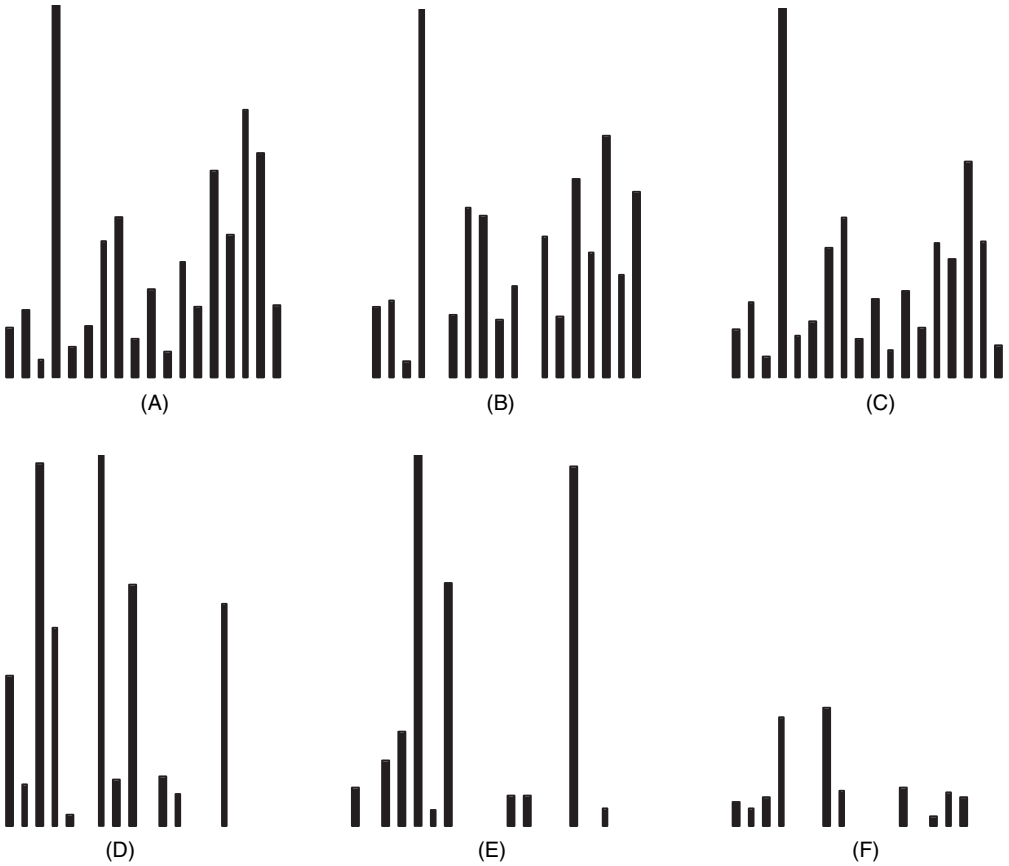

Figure 8. Bar coded HPLC fingerprints of different plant parts of Danshen. (A) IIDS; (B) IIDSM; (C) IIDSP; (D) IIDSF; (E) IIDSL; (F) IIDSR. (A) II *Salvia miltiorrhiza* Bge.; (B) II xylem; (C) II cortex; (D) II flower; (E) II leaves; (F) II branch.

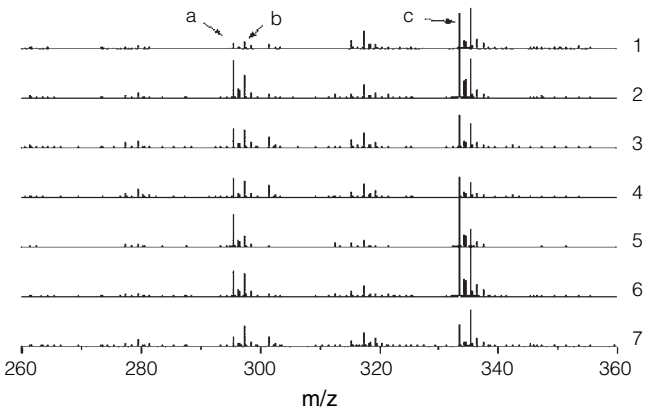

Figure 9. TOF/MS fingerprints of Danshen from different geographic locations in China. a: Tanshinone IIA [M+H]+; b: Cryptotanshinone [M+H]+; c: Tanshinone IIB [M+Na]+. 1: Jiangxi; 2: Zhongjiang Sichuan; 3: Henan; 4: Anhui; 5: Emei Sichuan; 6: Sichuan; 7: Beijing.

(PCs) are found to account for 98.0% of the variability in the samples, and are thus judged to be good enough to construct the model. A plot of score PC1 against PC2 for the NIR spectra of the two Danshen samples is shown in Figure 10 (B). The two plant species of Danshen are nicely separated in different clusters in the PC score plot. The PC scores of the samples can also be input to Hierarchical Cluster Analysis (HCA). The output of HCA is then plot as a tree-shaped dendrogramto that groups the samples according to similarities or dissimilarities quantified by distances. Many examples of HCA can also be found in the references listed in Table 9.

SIMCA (Soft Independent Modeling of Class Analogy) as a pattern recognition technique has also been used for the analysis of NIR fingerprints of Danshen samples collected from different geographical origins, produced under different growth conditions and of different plant parts. In SIMCA, typical modeling is carried out using some of the objects contained in a class training set, and the validity of the model is then tested on the remaining objects belonging to the same class (test set). Table 10 shows the performance of SIMCA analysis in this particular study and it is seen that most sample data in the three test sets can indeed be correctly identified.

CONCLUSIONS

The pharmaceutical actions of Chinese medicinal herbs often cannot be attributed to a particular compound

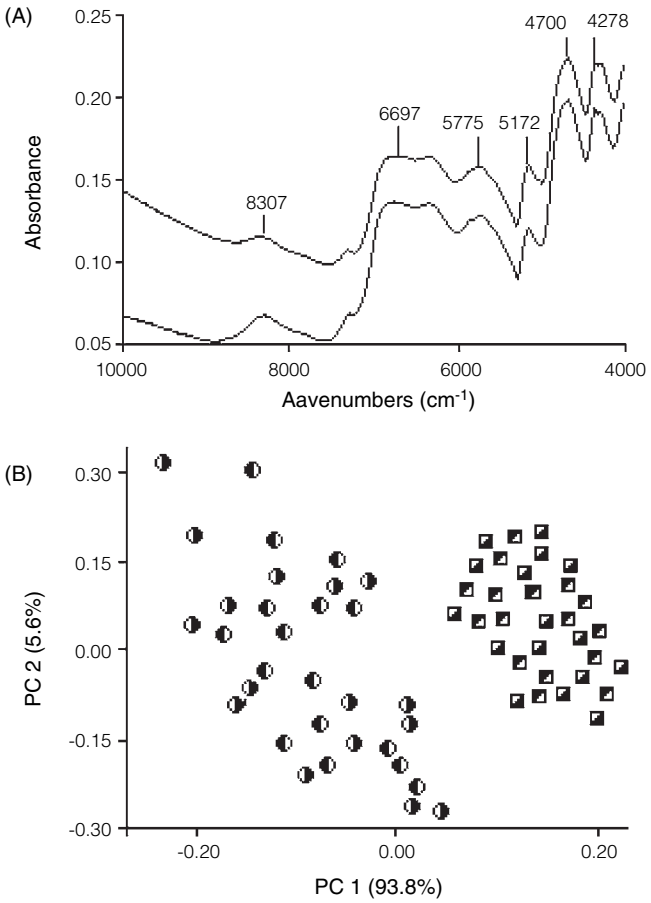

Figure 10. (A) NIR reflectance spectra of S. miltiorrhiza Bge (down) and S. paramiltiorrhiza (up) originated from Zhongjiang in Sichuan. (B) Plot of scores for the first and second principal components for the discrimination of S. miltiorrhiza Bge (◪ or ◩) and S. paramiltiorrhiza (◑ or ◐) originated from Zhongjiang in Sichuan using NIR reflectance spectra.

but rather to the synergy of compound mixtures (active fractions). Standardized extract of Herbs with quantified marker species and reproducible compositional fingerprint can serve as a standard reference material for the authentication and quality evaluation of herbal products. Standardization requires the control of all process variables in order to produced extracts with compositional uniformality, batch to batch consistency and consistent quality and property. These factors include the plant species, the plant part, the primary processing step, post plant harvest, the extraction procedure and the manufacturing process leading to the products. The extraction method should be selected and optimized based on two considerations: (1) it should contain at least the major and preferably all the active components in the original herb (2) it should reflect the efficacy of the original herb, preferably with defined potency or dose-response relationship through subsequent bioassays. Because of the complexity of ingredients in herbal drugs, more than one extract may be needed for adequately assessing quality.

Table 10. Performance of SIMCA modeling of NIR results

Sample	Test set	Valid	Misses
Geographical origins	21	18	3
Growing conditions	30	30	0
Parts of plant	24	18	6

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