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Review

Extraction methods and chemical standardization of botanicals and herbal preparations

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Abstract

Botanicals and herbal preparations are medicinal preparations, containing a single or two or more medicinal plants. The focus of this review paper is on the analytical methodologies, which included the combination of sample preparation tools and chromatographic techniques for the chemical standardization of marker compounds or active ingredients in botanicals and herbal preparations. The common problems and key challenges in the chemical standardization of botanicals and herbal preparations were discussed. As sample preparation is the most important step in the development of analytical methods for the analysis of constituents present in botanicals and herbal preparations, the strength and weakness of different extraction techniques are discussed. For the analysis of compounds present in the plant extracts, the applications of common chromatographic techniques, such as HPLC, CE, HRGC/MS, HPLC/MS and HPLC/MS/MS are discussed. The strength, weakness and applicability of various separation tools are stated. Procedures for the identification of marker or active compounds in plant extracts, using HPLC/MS, were proposed. Finally, the effects of batch-to-batch variation of the medicinal plants are investigated and discussed.

Keywords: Sample preparation; Extraction; Botanicals; Herbal preparations

Contents

1. Introduction

Botanicals and herbal preparations are medicinal preparations, containing a single plant or a mixture of two or more different types of medicinal plants. Monographs of medicinal plants can be found in the United States Pharmacopeia [1], Chinese Pharmacopeia [2], WHO monographs for medicinal plants [3,4], Japanese Pharmacopeia [5] and Herbal Medicine (Expanded Commission E Monographs) [6]. Other information on the chemical substances in medicinal plants and their pharmacological properties can be found in the

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Table 1
Some of the common herbal products, their uses and side effects [3,6]

Botanicals	Common medicinal uses	Side effects
Aloe vera	Short-term treatment of occasional constipation	Abdominal spasms and pain may occur after even single dose. Overdose can lead to colicky abdominal spasms and pain, as well as the formation of thin, watery stools. Overdose will result in electrolyte imbalance.
Echinacea	To support and promote the natural powers of resistance of the body, especially in infectious conditions, such as influenza and cold in the nose and throat. External uses include promotion of wound healing and treatment of inflammatory skin conditions.	Chills, short-term fever reaction and nausea and vomiting may occur.
St. John's Wort	For psychovegetative disturbances, depressive moods, anxiety and nervous unrest	Photosensitization is possible, especially in fair skin individuals.
Gingko biloba	For symptomatic treatment of disturbed performance in organic brain syndrome within the regimen of a therapeutic concepts, with the following principal symptoms: memory deficients, disturbances in concentration, depressive emotional conditions, dizziness, tinnitus and headache.	Very seldom, cases of stomach or intestinal upset, headaches or skin allergic skin reaction.
Galic	As a support to dietary measures at elevated levels of lipids in the blood and as a measure for age dependant vascular changes.	In rare instances, there may be gastrointestinal symptoms, changes to the flora of the intestine, or allergic reaction.
Ginseng	A tonic for invigoration and fortification times of fatigue and debility or declining capacity for work and concentration.	None
Liquorice root	For bronchitis, peptic ulcer, chronic gastritis, rheumatism and adrenocorticoid insufficiency.	On prolong use and higher doses, sodium and water retention and potassium loss may occur, accompanied by hypertension, edema, hypokalemia and in rare cases, myoglobinuria.

books cited [7–10]. A list of commercially available products, containing medicinal plants, their common medicinal uses and side effects are tabulated in Table 1. The major compound types in Chinese medicinal plants include alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins.

Botanicals, which show promising results included the use of *Ginkgo biloba* in which the gingkolides have antioxidant, neuroprotective and chlolinergic activities relevant to Alzeimer's disease [11–13]. The in vivo neuromodulatory effects of *G. biloba* was studied, using a product that is a standardized leaf extract (EGb761) reported to contain 24% flavone glycosides (primarily composed of quercetin, kaempferol and isorhamnetin) [13]. The inhibitory effects of cancer cell proliferation and anti-tumor effects of the total extracts from *Scutellariae radix* were well studied. In an in vivo experiment, significant inhibition of tumor growth had been observed. *Scutellaria baicalensis* inhibited cyclooxygenase-2 (COX-2) expression and a 66% reduction in tumor mass was observed in the nude mice. *S. baicalensis* selectively and effectively inhibits cancer cell growth in vitro and in vivo and can be an effective chemotherapeutic agent for head and neck squamous cell carcinoma. It was proposed that the inhibition of PGE_2 synthesis via suppression of COX-2 expression may be responsible for its anticancer activity [14,15]. The pharmacological basis of 'Yang-invigoration' in herbs, such as *Herba epimedii*, *Rhizoma drynariae* and others were discussed [16]. On the other hand, problems with regard to the usage of herbs were encountered. Chinese herb nephropathy or end stage renal failure was reported in patients taking weight reducing pills containing Chinese herbs, because of a manufacturing error, one of the medicinal plants in the pills was replaced with *Aristolochia fangchi* where the main components are aristolochic acids [17]. In Singapore, undeclared drugs, such as chlorpheniramine, paracetamol and others can be found in health supplements and herbal preparations [18].

At the same time, works with some botanicals have progressed to clinical trials. A multicentric, open, prospective, observational and non-randomized clinical trial with 190 patients for the efficacy and safety of a standardized phytoestrogen preparation derived from *Glycine max* (L.) Merr in Climacteric symptomatology was carried out [19]. A randomized controlled trial with 428 patients on the effects of standardized *Hypericum perforatum* (St. John Wort) in major depressive disorder was reported [20]. A Phase II clinical trial on botanical explored standardized green tea's antineoplastic effects in patients with androgen independent prostate carcinoma [21]. Other Phase II and III clinical trials sponsored by National Center for complementary and alternative medicine (NCCAM) are progressing at the moment (http://nccam.nih.gov).

For botanicals and herbal preparations, there is a need to approach scientific proof and clinical validation with chemical standardization, biological assays, animal models and clinical trials. Quality assurance of botanicals and herbal preparations is the prerequisite of credible clinical trials. According to the draft guidelines stated by the United States Food and Drug Administration (USFDA) [22] and The European Agency for the Evaluation of Medicinal Products [23], various aspects of analysis must be performed for the purpose of certification of botanical drugs and herbal preparations. For medicinal plants, the tests include identification, water

content, chemical assay of active ingredients, inorganic impurities (toxic metals), microbial limits, mycotoxins, pesticides and others. For herbal preparations, in addition to the tests mentioned above, other tests include disintegration, dissolution, hardness/friability and uniformity of dosage unit should be available. However, due to the unique nature of botanicals and herbal preparations, the USFDA found it appropriate to apply regulatory policies that was different from those applied to synthetic, semi-synthetic, or otherwise highly purified or chemically modified drugs. Clinical studies may be initiated at phase II for common botanicals. The continuing drive to optimize health in today's society has created a huge market for dietary supplements where there is a growing need to validate methods of analysis in botanicals and herbal preparation [24].

The focus of this review paper is on the analytical methodologies for the chemical standardization of marker compounds or active constituents in botanicals and herbal preparations. According to draft guidelines stated by the USFDA, a marker compound is a chemical constituent of a botanical raw material, drug substance, or drug product that is used for identification and/or quality control purposes, especially when the active constituents are not known or identified. The active constituent is responsible for the intended pharmacological activity or therapeutic effects. Chemical standardization often involves chemical identification by spectroscopic or chromatographic fingerprint and chemical assay (assay) for active constituents or marker compounds if available. The key challenges in the development of analytical methods for botanicals and herbal preparations are: (1) analysis of marker or active compounds in a complex and sometimes unknown environment, (2) target analytes may be polar and thermally labile, (3) lack of chemical reference substances and certified reference materials, (4) selection of extraction method and (5) batch-to-batch variation of the composition of the plant materials obtained. The analytical methods developed should be stability indicating, be used for chemical fingerprinting and assaying of marker or active compounds. With the chemical fingerprint obtained, the method should be able to perform composition analysis and monitor the batch-to-batch variation of the plant materials obtained for use. Due to the number of parameters required, methods that are simple, rapid and environmentally friendly are attractive options for regulatory laboratories, testing laboratories and laboratories supporting manufacturing activities. For the analysis of botanicals and herbal preparations, a holistic solution involving the combination of the methods of extraction and analytical techniques, such as separation tools are required.

In this article, recent developments and applications of modern sample preparation techniques and separation tools for final analysis in plant extracts are reviewed. Emphasis is placed on the brief description of the unique capabilities and advantages and disadvantages of the approaches used. The common problems and challenges in the analysis of target components in botanicals and herbal preparations will be discussed. More detailed description of the basic principles of these modern sample preparation tools, chromatographic techniques and hyphenated procedures, can be found in the review articles and other papers cited in the respective sections.

2. Preparation of sample

Sample preparation is the most important step in the development of analytical methods for the analysis of botanicals and herbal preparations. Review papers on sample preparation techniques for the extraction plant materials are available [25,26]. A brief summary of the experimental conditions for the various methods of extraction stated in this paper can be found in Table 2. The basic operation included steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analyte extraction. For the monograph stated in the pharmacopeia, methods, such as sonication, heating under reflux, Soxhlet extraction and others are commonly used [1,2,5]. However, such methods can be time consuming, required the use of large amount of organic solvent and may have lower extraction efficiencies. As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. The other problem for the selection of methods of extraction is that the active or marker compounds are present naturally, significant analyte–matrix interaction will be present, hence spiking of the target compounds into the plant matrix will not mimic the real environment. Depending on how the method is validated, it may be possible to have a method with high recovery but lacks accuracy. From the various botanicals studied in our laboratory, different methods of extraction and differing conditions may often be required for the extraction of marker compounds from different plant materials. Even with the same technique of extraction, for different marker compounds in different plant materials, different operating conditions, such as the solvent use, temperature applied and others may be required.

In the move to reduce or eliminate the use of organic solvent and improve the extraction processes, newer sample preparation methods, such as microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) have been introduced for the extraction of analytes present in plant materials. Using MAE, the microwave energy is used for solution heating and results in significant reduction of extraction time (usually in less than 30 min). Other than having the advantage of high extraction speed, MAE also enables a significant reduction in the consumption of organic solvents [25–27]. A novel MAE method had been developed for the extraction and determination of tanshinones (tanshinone IIA, cryptotanshinone and tanshinone I) from the root of *Salvia miltiorrhiza* with analysis by HPLC [28,29]. Other methods, such as the use of SFE that used carbon dioxide and some form of modifiers had been used

Table 2
A brief summary of the experimental conditions for various methods of extraction, such as sonication, Soxhlet extraction, MAE, supercritical extraction, ASE, PLE, PHWE and surfactant assisted PHWE for medicinal plants

	Sonication	Soxhlet extraction	Microwave assisted extraction (MAE)	Supercritical Fluid extraction (SFE)	Accelerated Solvent extraction, static (ASE)	Pressurized Liquid Extraction, dynamic (PLE)	Pressurized Hot Water Extraction (PHWE)	Surfactant assisted PHWE
Common Solvents used	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Carbon dioxide or carbon dioxide with modifiers, such as methanol	Methanol	Methanol	Water or water with 10–30 % ethanol	Water with surfactants, such as Triton X100 or SDS
Temperature (°C)	Can be heated	Depending on solvent used	80–150	40–100	80–200	80–200	80–300	80–200
Pressure applied	NA	NA	Depending on if it is closed or opened vessel extraction	250–450 atm	100 bar	10–20 bar	10–50 bar	10–20 bar
Time required	1 h	3–18 h	10–40 min	30–100 min	20–40 min	20–40 min	40–50 min	40–50 min
Volume of solvent required (ml)	50–100	150–200	20–50	NA	20–40	20–30	40–45	40–45
Refs.	[25,26]	[25,26]	[25–29]	[25,26,30,31]	[25,26,32]	[34–40]	[38,41–46]	[45,48]

in the extraction of compounds present in medicinal plants [25,26,30,31].

In the move to reduce the use of solvent and time in the methods of extraction, our laboratory have developed analytical methods for botanicals and herbal preparations using PLE. Methods using PLE, have been commonly used for the extraction of persistent organic pollutants in environmental samples. The technique uses organic solvent at elevated temperature and pressure, which drastically improves the speed of the extraction process. The parameters that have a significant effect on the extraction efficiencies of marker compounds in botanicals and herbal preparations by PLE are the time for extraction/volume of solvent, applied temperature and the nature of solvent used. The time for extraction was set at 20 min as it was found that a significant portion of the target analytes would be extracted. The pressure was observed to have little effect on the extraction efficiency as it was applied to keep the solvent in the liquid phase. Application of PLE, using commercially available systems based on static extraction to medicinal plants, was reported [32,33]. Our laboratory have reported the used of a laboratory made dynamic PLE system for the extraction of aristolochic acids, berberine, strychnine, ginsenosides, glycyrrhizin, baicalein and others in medicinal plants and herbal preparations [34–38]. It was observed that the extraction efficiency of marker compounds in botanicals and herbal preparations by PLE was comparable or higher than Soxhlet extraction. The main reasons for the enhanced performance when using PLE over Soxhlet extraction and other conventional methods of extraction are the higher solubility of analytes in solvent and higher diffusion rate as a result of higher temperature. At higher temperature, the strong solute–matrix interaction caused by van der Waals forces, hydrogen bonding and dipole attractions between solute molecules and active sites on the matrix were disrupted. The used of pressurized solvent extraction of polar steroids, such as withanolides from the leaves of *Lochroma gesnerioides* and cocaine and benzylecgonine from coca leaves had been reported [39,40].

Water has similar properties as methanol at an applied pressure of 50 bar and a temperature between 150 and 200 °C. However, it was observed that a number naturally occurring substances may decompose, using the stated temperature. To reduce the use of organic solvent, pressurized hot water extraction (PHWE) was used for the extraction of essential oil components from plant materials was developed [41,42]. Sub-critical water under pressure and between 125 and 175 °C had been used to rapidly extract oxygenated fragrance and favor compounds from rosemary [43]. A laboratory made system using sub-critical water was developed for the extraction of iridoid glycosides in plant matrix with final determination by micellar-electrokinetic capillary chromatography [44]. In our laboratory, PHWE was applied for the extraction of thermally labile and reasonably polar components, such as berberine in *Coptidis rhizoma*, glycyrrhizin in *Radix glycyrrhizae* (liquorice), baicalein in *S. radix* and marker compounds in *Radix codonopsis pilosula* [38,45]. An

instrumental set-up similar to PLE can be used for PHWE. The parameters that would have a significant effect on the extraction efficiencies of marker compounds in botanicals, using PHWE, are the applied temperature, pressure and percentage of organic modifiers. The non-significant effect of the pressure was observed.

However, due to the heat sensitivity and unique properties of certain marker compounds in botanicals, the extraction efficiencies of PHWE may not be as comparable to conventional methods, such as Soxhlet extraction. For components, such as tanshinone I and IIA in *S. miltiorrhiza*, PLE using methanol was found to give comparable or higher extraction efficiencies compared to PHWE with reference to Soxhlet extraction [46]. For the analysis of marker compounds in *R. codonopsis pilosula* (DangShen), it was observed that PHWE give lower extraction efficiencies compared to Soxhlet extraction [45]. To improve the extraction efficiencies of methods using PHWE, surfactants, such as Triton X-100 and sodium dodecyl sulfate (SDS) may be added into the water to enhance the solubility of target compounds present in the botanicals. In this model of extraction, a high dilution of the extract was produced as higher extractant volume was used. With surfactant assisted PHWE in a dynamic mode, the presence of surfactant, such as Triton X-100 in the water was found to enhance the solubility of the target compounds and pushed the target analytes in the sample matrix in the mobile phase to completeness with the fresh liquid pumped through the sample continuously [45]. Hence, micelle-mediated extraction and preconcentration of ginsenosides from Chinese herbal medicine had been introduced. When compared to methanol and water, an aqueous surfactant solution containing 10% Triton X-100 yielded faster kinetics and higher recovery for the extraction of various ginsenosides [47]. A method, using the combination of surfactant and PWHE with an applied temperature below the boiling point and lower pressure from 10 to 20 bars was developed for the analysis of marker compounds that are reasonably hydrophobic, such as tetradeca-4E, 12E-diene-8,10-diyne-1,6,7-triol and tetradeca-4E, 12E-diene-8-10-diyne-1,6,7-triol-*O*-β-D-glucoside in *R. codonopsis pilosula* (DangShen). The type of surfactants, such as Triton X-100, sodium dodecyl sulfate (SDS) and others added to the water will affect the extraction efficiencies of the methods using surfactant assisted PHWE [45]. Similarly, PLE with nonionic surfactants solution had been applied for the extraction of ginsenosides from the roots of American ginseng as model solid samples. The combination of PLE and cloud point extraction was shown to be a new and effective approach for the rapid sample pre-concentration of herbal materials prior to analysis by HPLC [48].

3. Analysis of botanicals and herbal preparation by liquid chromatography

Liquid chromatography with a isocratic/gradient elution remain to be the method of choice in the pharmacopeia and based on the number papers cited in this review for the analysis of marker compounds that are thermally labile in botanicals and herbal preparations. In most of the papers cited, the reversed octadecyl silica (C-18) is most commonly used. In the course of our experiments, we found that columns with smaller inner diameter, such as 1.0 or 2.1 mm i.d. were well suited to the analysis of components present in botanicals. For columns with smaller inner diameter, it was observed that the system precision for the retention time and peak area/height were comparable to analytical columns with 4.6 mm i.d. [46]. Most important of all, methods using columns with smaller inner diameter and the right mobile phase can be readily adopted to mass spectrometry. The most common mode of detection remains to be ultraviolet detection. Applications of chromatographic techniques to medicinal plants and Chinese traditional medicines are outlined [49]. Methods using gradient elution HPLC with reversed phase columns had been applied for the analysis of multiple constituents present in medicinal plants and herbal preparations [50–52]. Gradient elution HPLC with ultraviolet detection, using a C18 reversed phase column had been used to profile components present in *C. rhizoma*, *Radix aristolochiae*, ginseng, *R. glycyrrhizae* (liquorice), *S. radix*, *R. codonopsis pilosula* and *S. miltiorrhiza* [34,36,38,45,46]. The advantages of liquid chromatography include its high reproducibility, good linear range, ease of automation and its ability to analyze the number of constituents in botanicals and herbal preparation. However, for the analysis of marker compounds in herbal preparations with two or more medicinal plants, co-eluting peaks were often observed in the chromatograms obtained due to the complexity of the matrix [54]. The complexity of matrix may be reduced with additional sample preparation steps, such as liquid-liquid partitioning, solid phase extraction, preparative LC and TLC fractionation.

One of the challenges for the analytical methods developed was to study the effects of batch-to-batch variations in the medicinal plants. It was reported that HPLC analysis of marker compounds in *Platycodi radix* of *Playcodon grandiforum* showed variation in the composition from different sources [55]. With HPLC methods for simultaneous analysis of cichoric acids and alkamides in *Echinacea purpurea* plant and products, a selection of international herbal products available on the Danish market show surprising variable quality, not necessary reflecting the product information given on the labels [56]. A reversed phase HPLC method with ultraviolet detection was used to identify ginsenosides in products classified as health supplements where the results did not reflect what is given on the label [36]. Similarly, in one product labeled as containing pure *Panax ginseng*, no ginsenosides could be detected [8]. For ginseng, the variety and the method of cultivation are very important. It was observed that various species of ginseng, from a morphological point of view, are similar, but as far as their content of substances is concerned they are not [8].

To investigate the batch-to-batch variations of the plant materials obtained, a method using PLE with liquid

Table 3
Comparison of *Radix scutellariae* extracted from different batches of medicinal plants by PLE at 120 °C with HPLC [31]

Batch No.	Amount of Baicalein (mg/g)	EC50
1	20.65 ± 0.20	18.13 ± 0.75
2	21.47 ± 0.28	17.63 ± 1.65
3	12.80 ± 0.16	11.88 ± 0.25
4	19.34 ± 0.16	17.00 ± 0.58

For the determination of EC50, sample stock solutions were diluted to five different concentrations in ethanol. One ml of a 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solutions of different concentration and allowed to react at room temperature. After 30 min the absorbent values were measured at 518 nm, using a Jasco V-530 LSE 1335 UV Spectrophotometer (Japan) and converted into the percentage antioxidant activity (AA), using the following formula: $AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$. Ethanol (1.0 ml) plus plant extract solution (2.5 ml) was used as a blank. DPPH solution (1.0 ml; 0.3 mM) plus ethanol (2.5 ml) was used as a negative control. The positive controls were those using the standard solutions. EC50 represents the level where 50% of the radical were scavenged by test sample.

chromatography with ultraviolet detection [38] was used to assay the content of baicalein in *S. radix* from four different sources labeled as Batch 1–4 in Table 3. As shown in Table 3, batch-to-batch variations were observed with the amount of baicalein varied from 12.8 to 21.47 mg/g. A chromatographic fingerprint of various components present in *S. radix* was shown in Fig. 1. A similar chemical fingerprint as in Fig. 1 was obtained for all the four different batches of *S. radix* profiled. On top of the chemical fingerprint, the antioxidant activity, using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical photometric assay in the process guided by its discoloration, was measured. However, the level of baicalein and the antioxidant activities was found to vary in the four different batches of *S. radix*. The anti-oxidant activities measured were

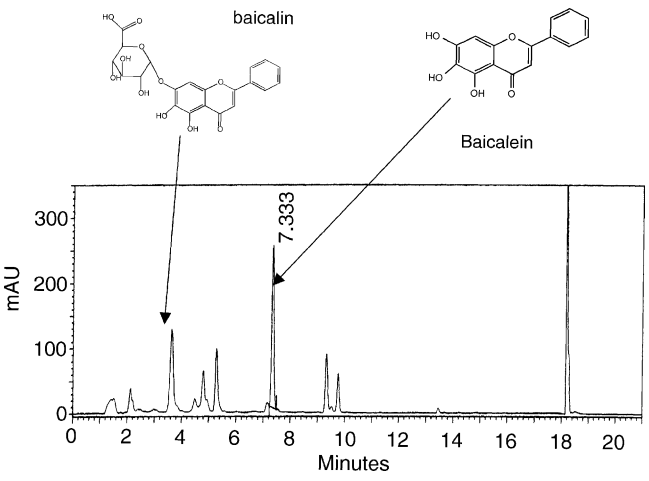

Fig. 1. HPLC Chromatogram obtained for baicalein in *S. radix* by PLE. Mobile phase of (A) 25 mM NaH_2PO_4 at pH 2.5 and (B) acetonitrile. Initial condition was set at 30% of B, gradient up to 100% B in 15 min before returning to initial condition for 10 min. C-18 reversed phase HPLC column (4.6 mm i.d. × 150 mm length, 5 μ. Detection was at 254 nm. Oven temperature was at 40 °C and flow rate was 1.0 ml/min.

found to correlate with the amount baicalein and other components present in *S. radix*. Hence, the assayed of marker or active compounds together with chemical fingerprinting, using HPLC, will be able to provide further information about the quality of the botanicals and herbal preparations.

4. Analysis of botanicals and herbal preparations by capillary electrophoresis

Capillary electrophoresis (CE) proved to be a powerful alternative to HPLC in the analysis of polar and thermally labile compounds. Reviews on the analysis of natural medicines or natural products in complex matrix by CE are well reported [56,57]. Many publications showed that all aspects of CE, such as capillary zone electrophoresis (CZE), micellar-electrokinetic capillary chromatography (MEKC) and capillary isoelectric focusing (cIEF) have been used for the separation of natural products. The separation in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer in the capillary. For MEKC, the main separation mechanism is based on solute partitioning between the micellar phase and the solution phase. Factors that are known to affect separation in CZE and MEKC include pH of running buffer, ionic strength, applied voltage and concentration and type of micelle added.

From the review articles, CE had been used to determine the amount of catechin and others in tea composition, phenolic acids in coffee samples and flavonoids and alkaloids in plant materials [56,57]. In our laboratory, analytical methods using CE, included the determination of aristolochic acids in *R. aristolochiae*, strychnine in *Strychnos nux-vomica*, berberine in *Rhizoma coptidis* (Huang-lian) and glycyrrhizin in *R. glycyrrhizae* (liquorice) [35,37,53]. CZE was found to be a method for the rapid separation and determination of four phenylpropanoid glycosides from *T. chamaedrys* [58]. Methods using CZE and MEKC have been proved to be a powerful technique in the chemical profiling and assaying of marker compounds present in extracts from medicinal plants.

As herbal preparations are products that were often observed to contain two more different types of medicinal plants, it had always been a challenge for the analysis of target compounds in herbal preparations and products that are classified as, Traditional Chinese Medicine (TCM) or herbal remedies. It is certain that the presence of the number of medicinal plants used in the herbal preparations will increase the complexity of the sample matrix obtained. In the analysis of marker compounds in complex environment, methods using MEKC had been applied for the determination of icariin, rhein, chrysophanol, physcion, glycyrrhetic acid and glycyrrhizic acid in traditional Chinese herbal preparations [59]. The use of MEKC with electrochemical or ultraviolet detection had been described for the determination of hesperidin, naringin, puerarin and

daidzein in medicinal preparations [60,61]. To differentiate between medicinal plant, such as *S. radix* from *Astragali radix*, CE with electrochemical detection was employed [62].

For the determination of berberine, aristolochic acids I and II in herbal preparations, we reported that it was possible to assay the amount of marker or active compounds with a single step extraction followed by CZE. This was due to the fact that separation by CZE is mainly based on the different electrophoretic mobility of charged solutes in solution in relation to their different molecular weight, size and charge at a given pH. It is also possible for both cations and anions to be separated in a single run which provides specific selectivity for the separation method. It was observed that it can be difficult to assay the amount of marker compounds in herbal preparations using HPLC as co-eluting peaks were often present. With CZE and under the conditions used for aristolochic acids, it was found that most of the positively charged and neutral species migrate close or with the electroosmotic flow (EOF). The amount of aristolochic acid I and II present in herbal preparations, that was in a complex environment, could be determined with a single step extraction followed by CZE [35,53].

The advantages of methods using CE for botanicals and herbal preparations are: (1) the high selectivity and ability to analyze target compounds in complex environment, such as herbal preparations without additional sample preparation steps, (2) significant reduction or complete elimination of organic solvent for the electrolyte solution or running buffer, (3) good linear range, (4) ease of automation and (5) speed of analysis. Using ultraviolet detection, on-column detection was often used in CE. Without using bubble capillary columns and applying any specific pre-concentration techniques, this will result in lower sensitivity compared to methods using HPLC. To improve the reproducibility of migration time and peak area with methods using CE, internal standards are often added. However, the system precision for the relative migration time and peak area will always be higher in methods using CE compared to HPLC. It was observed in our laboratory that run failures were encountered in CZE and MEKC where standard solutions were prepared in high percentage of methanol. The effect was due to a disruption of the conductivity inside the capillary caused either by the presence of a non-conductivity plug or the result of the out-gassing in the injection zone. As the sample was extracted with methanol, 10% water in methanol was added to standard solutions and sample extracts to counter run failures [35,37,53]. Our observations on run failure, was consistent with other reports [63,64].

Despite of some the weakness described for methods using CE, the enantioseparation of naturally occurring substances, such as atropine and flavonoids, such as medicarpin and vestitone in plant materials by CE with chiral selectors remained to be the method of choice [65–67]. From our works with plant materials, the applicability of methods using CE, will be more difficult for compounds that are essentially non-polar present in some medicinal plants.

5. Hyphenation procedures

The use of chromatographic separation with mass spectrometry for the chemical characterization and composition analysis of botanicals has been growing rapidly in the recent years. Reviews on the use of mass spectrometry and high-performance liquid chromatography mass spectrometry (HPLC/MS) on botanicals had been reported [68,69]. The use of hyphenated techniques, such as high resolution gas chromatography mass spectrometry (HRGC/MS), high performance liquid chromatography/mass spectrometry (HPLC/MS), liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and tandem mass spectrometry (MS/MS) to perform on line composition and structural analyses provide rich information that is unsurpassed by other techniques.

The use of HRGC/MS, remains the method of choice for the analysis of volatile and semi-volatile components, such as essential oil and others in botanicals and herbal preparations. With high resolution separation with capillary column coupling with mass spectrometry using electron impact ionization (EI), undeclared drugs, such as chlorpheniramine, paracetamol and others that may be present or added in health supplements and herbal preparations can be detected easily [18]. Other than undeclared drugs, marker compounds, such as tetrahydrompalmatine in *Corydalis yanhusuo* can be identified using HRGC/MS with the chromatograms and the mass spectra as shown in Fig. 2. The power of HRGC/MS is the ability to rapidly detect compounds that are volatile or semi-volatile present in a complex environment, such as extracts from herbal preparations using a single step extraction without any additional clean-up steps.

For the analysis of components present in botanicals, HPLC/MS has been playing a increasingly significant role as the technique is capable of characterizing compounds that are thermally labile, ranging from small polar molecules to macromolecule, such as peptides/proteins, carbohydrates and nucleic acids. The most common mode of ionization in HPLC/MS included electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Mass analyzer, such as single quadruple, triple quadruple, ion-trap, time-of–flight, quadruple time-of-flight (Q-TOF) and others are used. With tandem mass spectrometry, additional structural information can be obtained for the target compounds. However, methods using HPLC/MS is still limited to conditions that are suitable for MS operations. There are restrictions on pH, solvent choice, solvent additives and flow rate for LC in-order to achieve optimal sensitivity.

In our laboratory and other report, HPLC/MS had been used to characterize ginsenosides in ginseng, tanshinone I, tanshinone II and cryptotanshinone present in *S. miltiorrhiza* [46,68] and marker compounds present in *R. codonopsis pilo-*

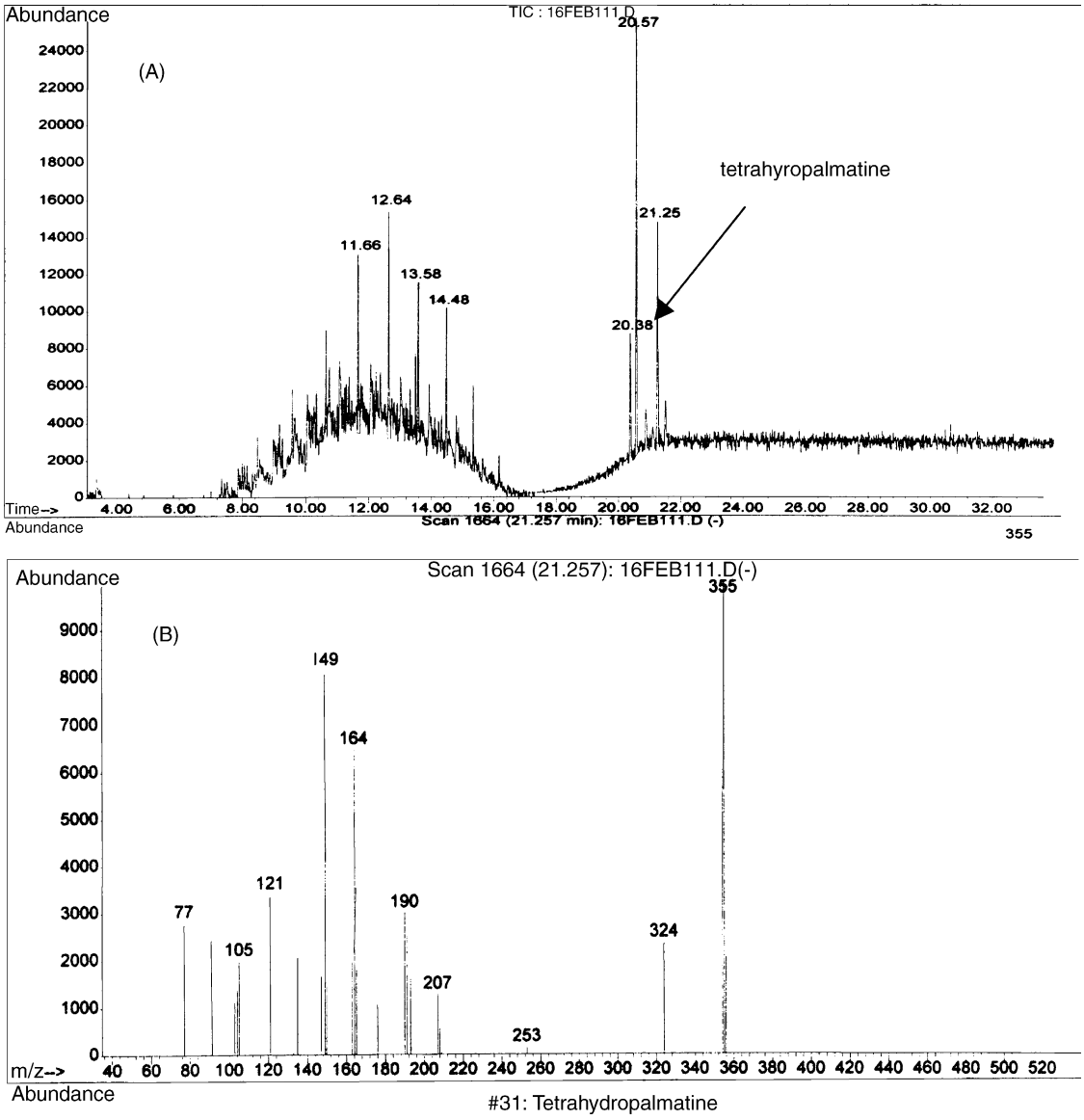

Fig. 2. A Total ion chromatograms (TIC) obtained for marker compounds, such as tetrahydrompalmatine in *C. yanhusuo* with HRGC/MS and 2B) mass spectra with electron impact (EI) ionization obtained for tetrahydropalmatine. Injector: splitless at 250 °C. Carrier: helium. Oven: initial at 80–300 °C at 15 °C/min. Detector: Mass selective detector, 280 °C, full scan: 40–600 amu. Column: HP5, 30 m × 0.25 mm i.d., 0.25 μm.

sula [45]. The presence of non-volatile components, such as tanshinone I, tanshinone II and cryptotanshinone in *S. miltiorrhiza* can be easily identified by positive ion ESI/MS. Direct sample introduction by ESI or separation with reversed phase column resulted in the abundant of $[M + H]^+$, $[M + Na]^+$ and $[M + M + Na]^+$ for tanshinone I, tanshinone II and cryptotanshinone [46,68]. For compounds, such as tetradeca-4E, 12E-diene-8,10-diyne-1,6,7-triol and tetradeca-4E, 12E-diene-8-10-diyne-1,6,7-triol-*O*-β-D-glucoside in *R. codonopsis pilosula* (DangShen), $[M + Na]^+$ was observed to be the most abundant ion [45]. With ESI/MS, there is a great tendency for certain compounds in medicinal plants to form adducts, such as $[M + Na]^+$ or $[M + K]^+$.

Methods using HPLC/ESI/MS had been applied for the identification components present in *Betula pubescen* and *Peschiera fuschiaefolia*. [70,71]. For the characterization of hydrolysable tannins from leaves of *B. pubescen*, the main peak in the mass spectra of an individual hydrolysable tannins in the negative ion ESIMS, was the deprotonated molecule $[M - H]^-$ [70]. The alkaloids, such as voacamine, voacamidine, vobasine and voachalotine present in *P. fuschiaefolia* were determined using external standard calibration with acceptable precision and accuracy. The presence of $[M + H]^+$, $[M + 2H]^{2+}$ and other adduct ions were observed in the positive electospray ionization mode [71]. Using APCI interface with mass analyzer, such as ion trap or triple quadruple instruments, main constituents present in *Rhodiola rosea* extracts and tropane alkaloids from *Erythroxylum vacciniifolium* can be characterized [72,73]. With internal standard calibration, constitutents, such as salidroside,

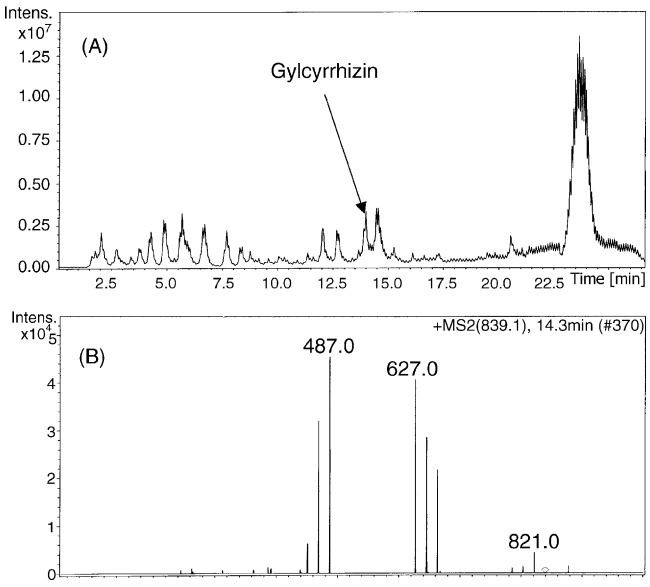

Fig. 3. A Total ion chromatogram (TIC) obtained for glycyrrhizin in the methanolic extract *R. glycyrrhizae* using HPLC/ESI/MS and 3B MS/MS of glycyrrhizin with precursor ion *m/z*: 839.1 amu. The gradient elution, using a mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The initial condition was set at 20% of B, gradient up to 100% in 20 min and returning to initial condition for 6 min. Oven temperature was set at 40 °C and flow rate was set at 0.2 ml/min. For all experiments, 5 μl of standards and sample extract were injected. The column was a reversed phase C18 Luna, 150 mm × 2.0 mm, 5 μm (Phenomenex, USA). The ESI-MS was acquired in the positive ion mode. The scanning mass range was from 100 to 1000 amu. The heated capillary temperature was maintained at 350 °C, the drying gas and nebulizer nitrogen gas flow rates were 10 l/min and 50 psi, respectively. Data was acquired using automated MS/MS.

rosavin and benzyl-*O*-β-glucopyranoside in *R. rosea* extracts was determined [73]. Similarly, the content of ginsenosides, such as Rb1, Rc and Re in plant extracts from *P. ginseng* and *Panax quinquefolius* was determined by HPLC/ESI/MS with external standard calibration. The $[M + H]^+$ and $[M + Na]^+$ ions were observed for ginsenosides standards (Rb1, Rb2, Rc, Rd, Re, Rf and Rg1) [74]. In our earlier work, it was observed that it would be difficult to differentiate between *P. ginseng* and *P. quinquefolius* using reversed phase HPLC with ultraviolet detection [36]. With HPLC/ESI/MS, the presence and ratio of ginsenosides Rf and 24-(R)-pseudoginsenosides F_{11} in the plant extracts can be used to differentiate between *P. ginseng* and *P. quinquefolius* [75,76].

From Figs. 3A and 4A, the total ion chromatograms (TIC) of glycyrrhizin and baicalein in the methanolic extracts from *R. glycyrrhizae* and *S. radix* were obtained, using HPLC/ESI/MS. With tandem mass spectrometry (MS^2), significant fragmentation pattern was observed for glycyrrhizin and baicalein as in Figs. 3B and 4B. In most instances, significant fragmentation patterns can be obtained for a number of compounds in plant materials with MS^2. However, for certain compounds, such as baicalin in *S. radix*, significant fragmentation pattern was not observed with MS^2 as in Fig. 4C. As baicalin and baicalein differs from each other by one side

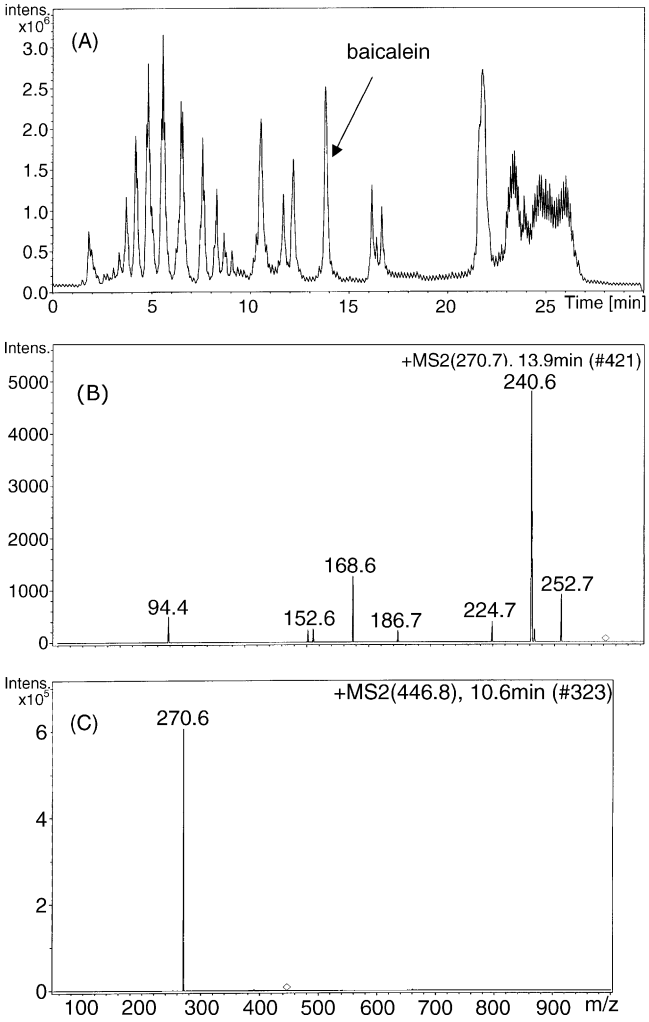

Fig. 4. A Total ion chromatogram (TIC) obtained for baicalein in the methanolic extracts from *S. radix* using LC/ESI/MS and 4B) MS/MS of baicalein with precursor ion *m/z*: 270.7 amu, 4C) MS/MS spectra of baicalin with precursor ion *m/z*: 446.6 amu. Conditions used were the same as in Fig. 3.

chain, MS/MS of balcalin with precursor ion *m/z*: 470.7 gave the molecular ion of baicalein as in Fig. 4C. With MS^3, the fragmentation pattern for baicalein as in Fig. 4B was observed for baicalin. The current example showed the ability of MS^2 and MS^3 in the structural elucidation of naturally occurring compounds in botanicals. For certain compounds, MS^2 may not be able to provide as much information compared to MS^3.

The strength of HPLC/MS and HPLC/MS^n remained in its ability to determine the presence of target compounds in complex environment, such as in herbal preparations. This was seen in the evaluation of major active components in St. John's Wort dietary supplements with HPLC/ESI/MS. The content of the target components, such as rutin, hyperosides, quercetin and others were determined using internal standard calibration. The presence of $[M + H]^+$ and $[M + Na]^+$ ions were observed for mass spectra obtained for rutin and hyperosides [77]. As a result of end stage

renal failure reported in patients taking weight reducing pills containing Chinese herbs that was found to contain aristolochic acids, the presence of aristolochic acids in a number of herbal remedies was screened and detected with methods using HPLC/ESI/MS and HPLC/APCI/MS [78]. Other groups have also used HPLC/MS to confirm the presence of aristolochic acids in Chinese phytomedicines and dietary supplements used as slimming regiments [79,80]. Using selective ion monitoring (SIM) or extracted ion chromatogram at a particular *m*/*z*, the mass analyzer can become a highly selective detector for the detection of target components in complex environment, such as herbal preparations. An HPLC/ESI/MS method had been developed for an estimation of the saponin content in food supplement containing *Tribulus terrestis L.* [81]. In conjunction with an AOAC task group on dietary supplement, a method using liquid chromatography/tandem mass spectrometry was validated for measurement of six major alkaloids in raw *Ephedra sinica* herb, *Ephedra* extracts and health supplement products [82]. In both methods, internal standard was added to determine the content of target compounds present.

For the identification of substances present in botanicals and herbal preparations using HRGC/MS or HPLC/MS, the authors' find that the following conditions are useful when standards are available. These include: (1) a suspect peak has to show retention time similar to average retention time of the pure standard or control sample and (2) mass spectra for the suspect peaks has to show relative abundance ± 10% (arithmetic difference) of the relative abundance of standard analyzed that day [83]. With HPLC/MS, applying the right separation, with the right ionization interface and mass analyzer, significant information can be obtained with regards to the target compounds. However, for the quantitation of compounds in plant materials, the system precision will be higher compared to HPLC with ultraviolet detection. For the analysis of target compounds in botanical extracts, systems that allowed for gradient elution HPLC are often required. For on-line HPLC/MS, the internal diameter of the column selected will be an important consideration. The used of 1.0 or 2.1 mm i.d. columns with flow rate at 100–200 μl/min or lower is optimal for most ESI/MS operation. Post column splitter is often needed to reduce LC flow into the mass spectrometer when using 4.6 mm i.d. columns with flow rate at 1.0 ml/min. On top of that, the common buffers, such as phosphate buffer and others used in most HPLC methods cannot be adopted for methods using HPLC/MS. The choice of buffers and ion pairing reagents that can be used in the proposed methods using HPLC/MS are more limited. For further information of the suitable of solvents and additives in HPLC/MS, the readers can refer to an excellent Ref. [84]. From our experiences, samples that are found to contain high salt content will create problems for the mass analyzer in most HPLC/MS system. Finally, interpretations of mass spectra from ESI/MS will be more tricky in the presence of adduct ions, such as $[M + Na]^+$, $[M + 2H]^{2+}$, dimmers and others.

6. Conclusions

Chemical analysis of extracts from plant material will play a central role in the development and modernization of Chinese medicines, botanicals and herbal preparations. The method used is required to identify the active or marker compounds, composition analysis and fingerprinting purposes. For the chemical standardization of botanicals and herbal preparations, the method involved sample preparation procedures, such as techniques of extraction and other analytical techniques, such as the separation tools. However, due to complexity of the plant materials obtained, different methods using different methods of extraction and separation tools may be needed. It is certain that methods that are simple, rapid and environmentally friendly will be preferred and is likely to play an important role in the effort in providing high quality products to consumers worldwide. Just as for pharmaceutical products, a well-validated method will help in monitoring the stability of the botanicals and herbal preparations over a period of time. It is likely that the combination of chemical standardization with biological assay will provide further knowledge about therapeutic effects of the medicinal plant. The use of validated methods in the chemical standardization of botanicals and herbal preparations will enhance the quality of the products, assist in pharmacological studies, perform credible clinical trials and propel the move towards evidence based medicine.

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