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Analytical Evaluation of Herbal Drugs

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1. Introduction

Traditional herbal medicine and their preparations have been widely used for the thousands of years in developing and developed countries owing to its natural origin and lesser side effects or dissatisfaction with the results of synthetic drugs. However, one of the characteristics of oriental herbal medicine preparations is that all the herbal medicines, either presenting as single herbs or as collections of herbs in composite formulae, is extracted with boiling water during the decoction process. This may be the main reason why quality control of oriental herbal drugs is more difficult than that of western drug. As pointed in "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines (World Health Organization, 2000)", "Despite its existence and continued use over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognized in most countries. Consequently, education, training and research in this area have not been accorded due attention and support. The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use world-wide. The reasons for the lack of research data are due to not only to health care policies, but also to a lack of adequate or accepted research methodology for evaluating traditional medicine" (WHO, 2000, 2001).

In olden days vaidas used to treat patients on individual basis and prepare drug according to the requirement of the patient but now the scene has changed, herbal medicines are being manufactured on large scale where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization methodology of single drugs and formulation, quality control parameters etc; hence the concept of quality from very first step is paramount factor must get good attention.

The chemistry of plants involves the presence of therapeutically important constituents usually associated with many inert substances (coloring agents, cellulose, lignin etc). The active principles are extracted from the plants and purified for therapeutic utility for their selective pharmacological activity. So quality control of herbal crude drugs and their constituents is of great importance in modern system of medicine. Lack of proper standard parameters for the standardization of herbal preparation and several instances of substandard herbs, adulterated herbs come into existence. To meet new thrust of inquisitiveness, standardization of herbals is mandatory (Chaudhry, 1999; Kokate, 2005; Raina, 2003; Raven, 1999; Yan, 1999).

Hence every single herb needs to be quality checked to ascertain that it confirms to quality requirement and delivers the properties consistently. Standardization assures that products are reliable in terms of quality, efficacy, performance and safety. It is however observed that the drugs in commerce are frequently adulterated and do not comply with the standards prescribed for authentic drug.

2. Drug adulteration

The adulteration and substitution of herbal drugs is the burning problem in herbal industry and it has caused a major effect in the commercial use of natural products. Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products. Adulteration it is a practice of substituting the original crude drug partially or fully with other substances which is either free from or inferior in therapeutic and chemical properties or addition of low grade or spoiled drugs or entirely different drug similar to that of original drug substituted with an intention of enhancement of profits. Or adulteration may be defined as mixing or substituting the original drug material with other spurious, inferior, defective, spoiled, useless other parts of same or different plant or harmful substances or drug which do not confirm with the official standards [Ansari, 2003; Kokate, 2004].

Adulteration may takes place by two ways:

- Direct or intentional adulteration
- Indirect or unintentional adulteration

2.1 Direct or intentional adulteration

Direct or intentional adulteration is done intentionally which usually includes practices in which an herbal drug is substituted partially or fully with other inferior products. Due to morphological resemblance to the authentic herb, many different inferior commercial varieties are used as adulterants. These may or may not have any chemical or therapeutic potential. Substitution by "exhausted" drugs entails adulteration of the plant material with the same plant material devoid of the active constituents. This practice is most common in the case of volatile oil-containing materials, where the dried exhausted material resembles the original drug but is free of the essential oils. Foreign matter such as other parts of the same plant with no active ingredients, sand and stones, manufactured artifacts, and synthetic inferior principles are used as substitutes.

The practice of intentional adulteration is mainly encouraged by traders who are reluctant to pay premium prices for herbs of superior quality, and hence are inclined to purchase only the cheaper products. This encourages producers and traders to sell herbs of inferior quality.

2.1.1 With artificially manufactured materials

Substances artificially manufactured being resemble with original drug are used as substitutes. This practice is generally followed for much costlier drug e.g. nutmeg is adulterated with basswood prepared to the required shape and size, the colored paraffin wax is used in place of beeswax.

2.1.2 With inferior quality materials

Inferior quality material may or may not have same chemical or therapeutic value as that of original natural drug due to their morphological resemblance to authentic drug, they are marketed as adulterants e.g. *Belladonna* leaves are substituted with *Ailanthus* leaves, *papaya* seeds to adulterate *Piper nigrum*, mother cloves and clove stalks are mixed with clove, beeswax is substituted by Japan wax.

2.1.3 With exhausted material

Many drugs extracted on large scale for isolation of active principle, volatile oils etc. the exhausted material may be used entirely or in part as a substituent for the genuine drug e.g. umbelliferous fruits and cloves (without volatile oils) are adulterated with exhausted (without volatile oils) original drugs, exhausted jalap and Indian hemp (without resins) are used as adulterant.

2.1.4 With foreign matter

Sometimes synthetic chemicals are used to enhance the natural character e.g. addition of benzyl benzoate to balsam of Peru, citral to citrus oils like oil of lemon and orange oil etc.

2.1.5 With harmful / Fictitious substances

Sometimes the wastes from market are collected and admixed with authentic drugs particularly for liquids or unorganized drugs e.g. pieces of amber colored glass in colophony, limestone in asafetida, lead shot in opium, white oil in coconut oil, cocoa butter with stearin or paraffin.

2.1.6 Adulteration of powders

Besides entire drug powder form frequently found to be adulterated e.g. powder liquorice or gentian admixed with powder olive stones, under the name of cinchona, *C. calisaya* wedd., *C. officinalis* Linn.f., *C. ledgeriana* and *C. succirubra* are available as mixtures.

2.2 Indirect or unintentional adulteration

Unintentional or undeliberately adulteration which sometimes occurs without bad intention of the manufacturer or supplier. Sometimes in the absence of proper means of evaluation, an authentic drug partially or fully devoid of the active ingredients may enter the market. Factors such as geographical sources, growing conditions, processing, and storage are all factors that influence the quality of the drug [Ansari, 2003; Kokate, 2004].

2.2.1 Faulty collection

Some of the herbal adulteration is due to the carelessness of herbal collectors and suppliers. The correct part of genuine plant should be collected. Other less valuable part of the genuine plant should not be collected. Moreover collection should be carried out at a proper season and time when the active constituents reach maximum. *Datura strumarium* leaves should be collected during flowering stage and wild cherry bark in autumn etc. collection from other

plant by ignorance, due to similarity in the appearance, color, lack of knowledge may lead to adulteration. For example in place of *Aconitum napellus*, the other *Aconitum deinorhizum* may be collected or in place of *Rhamnus purshiana* (cascara bark) *Rhamnus colifornica* is generally collected. Confusion existing in the common vernacular name of different plant in various states of india may leads to this type of adulteration. Often in different states the same plant is known by different vernacular names, while quite different drugs are known by same name. This creates confusion which is best illustrated by Punarnava and Brahmi. The Indian pharmacopoeia drugs *Trianthema portulacastrum* L. and *Boerhavia diffusa* L. are both known by the same vernacular name "Punarnava".

2.2.2 Imperfect preparation

Non removal of associated structures eg stems are collected with leaves, flowers, fruits. Non-removal of undesirable parts or structures e.g. cork should be removed from ginger rhizome. Proper drying conditions should be adhered. Improper drying may lead to unintentional adulteration e.g. if digitalis leaves are dried above 65^{o}c decomposition of glycosides by enzymatic hydrolysis. Use of excessive heat in separating the code liver oil from livers, where the proportions of vitamins, odor and color etc are adversely affected.

2.2.3 Incorrect storage

Deterioration especially during storage, leading to the loss of the active ingredients, production of metabolites with no activity and, in extreme cases, the production of toxic metabolites. Physical factors such as air (oxygen), humidity, light, and temperature can bring about deterioration directly or indirectly. These factors, alone or in combination, can lead to the development of organisms such as molds, mites, and bacteria. Oxidation of the constituents of a drug can be brought about by oxygen in the air, causing some products, such as essential oils, to resinify or to become rancid. Moisture or humidity and elevated temperatures can accelerate enzymatic activities, leading to changes in the physical appearance and decomposition of the herb. For example volatile oils should be protected from light and stored in well closed containers in cool place. Belladonna leaf should be stored in moisture free containers, which may cause enzymatic action lead to decomposition of medicinally active constituents. Mites, nematode worms, insects/moths, and beetles can also destroy herbal drugs during storage.

2.2.4 Gross substitution with plant material

Due to morphological resemblance i.e similarity in appearance, colors etc the genuine crude drugs are substituted with others are very often sold in the market e.g. *Podophyllum peltatum* L. is used as a substitute for *P. hexandrum*, *Belladona* leaves are substituted with Ailanthus leaves, saffron is admixed with dried flowers of *Carthamus tinctorius*, mother cloves and clove stalks are mixed with clove.

2.2.5 Substitution with exhausted drugs

In this type, the same drug is admixed but devoid of any medicinally active constituents as they are already extracted out. This practice is more common in case of volatile oil containing drugs like fennel, clove, coriander, caraway etc. sometime, natural characters of

exhausted drugs like color and taste are manipulated by adding other additives and then it is substituted eg exhausted gentian made bitter with aloes.

3. Drug evaluation

Evaluation means confirmation of its identity and determination of quality and purity of the herbal drug. Evaluation of crude drug is necessary because of three main reasons: biochemical variations in the drug, deterioration due to treatment and storage, substitution and adulteration as a result of carelessness, ignorance or fraud or variability caused by differences in growth, geographical location, and time of harvesting. For the quality control of a traditional medicine, the traditional methods are procured and studied, and documents and the traditional information about the identity and quality assessment are interpreted in terms of modern assessment or monograph in herbal pharmacopoeia [Ansari, 2003; Kokate, 2004; Gupta, 2007]. The crude drug can be evaluated or identified by five methods:

3.1 Organoleptic evaluation or morphological evaluation

It means evaluation of drug by the organs of sense (skin, eye, tongue, nose and ear) or macroscopic evaluation and it includes evaluation of drugs by color, odor, taste, size, shape and special feature, like touch, texture etc. it is the technique of qualitative evaluation based on the study of morphological and sensory profile of whole drugs. eg. The fractured surfaces in cinchona, quillia and cascara barks and quassia wood are important characteristics. Aromatic odour of umbelliferous fruits and sweet taste of liquorice are the examples of this type of evaluation where odor of drug depends upon the type and quality of odourous principles (volatile oils) present. Shape of drug may be cylindrical (sarsapilla), subcylindrical (podophyllum), conical (aconite), fusiform (jalap) etc, size represent length, breadth, thickness, diameter etc. color means external color which varies from white to brownish black are important diagnostic characters. The general appearance (external marking) of the weight of a crude drug often indicates whether it is likely to comply with prescribed standard like furrows(alternate depression or valleys), wrinkles (fine delicate furrows), annulations (transverse rings), fissures (splits), nodules (rounded outgrowth), scars (spot left after fall of leaves, stems or roots). Taste is specific type of sensation felt by epithelial layer of tongue. It may be acidic (sour), saline (salt like), saccharic (sweetish), bitter or tasteless (possessing no taste).

3.2 Microscopic evaluation

It involves detailed examination of the drug and it can be used to identify the organized drugs by their known histological characters. It is mostly used for qualitative evaluation of organized crude drugs in entire and powder forms with help of microscope [Ansari, 2003; Kokate, 2005; WHO, 1998].

Using microscope detecting various cellular tissues, trichomes, stomata, starch granules, calcium oxalate crystals and aleurone grains are some of important parameters which play important role in identification of certain crude drug. Crude drug can also be identified microscopically by cutting the thin TS (transverse section), LS (Longitudinal section) especially in case of wood and by staining them with proper staining reagents e.g. starch and hemicelluloses is identified by blue color with iodine solution, all lignified tissue give

pink stain with phloroglucinol and HCl etc. mucilage is stained pink with ruthenium red can be used to distinguish cellular structure. Microscopic evaluation also includes study of constituents in the powdered drug by the use of chemical reagents.

Quantitative aspects of microscopy includes study of stomatal number and index, palisade ratio, vein-islet number, size of starch grains, length of fibers etc which play important role in the identification of drug.

3.3 Chemical evaluation

Most of drugs have definite chemical constituents to which their biological or pharmacological activity is attributed. Qualitative chemical test are used to identify certain drug or to test their purity. The isolation, purification, identification of active constituents is based on chemical methods of evaluation. Qualitative chemical test such as acid value, saponification value etc. Some of these are useful in evaluation of resins (acid value, sulphated ash), balsams (acid value, saponification value and bester values), volatile oils (acetyl and ester values) and gums (methoxy determination and volatile acidity). Preliminary phytochemical screening is a part of chemical evaluation. These qualitative chemical tests are useful in identification of chemical constituents and detection of adulteration.

3.4 Physical evaluation

Physical constants are sometimes taken into consideration to evaluate certain drugs. These include moisture content, specific gravity, optical *rotation*, refractive, melting point, viscosity and solubility in different solvents. All these physical properties are useful in identification and detection of constituents present in plant.

3.5 Biological evaluation

Some drugs have specific biological and pharmacological activity which is utilized for their evaluation. Actually this activity is due to specific type of constituents present in the plant extract. For evaluation the experiments were carried out on both intact and isolated organs of living animals. With the help of bioassays (testing the drugs on living animals), strength of drug in its preparation can also be evaluated [Ansari, 2003; Kokate, 2005; Williamson, 1996]. Some important biological evaluations are as follow:

3.5.1 Antibiotic activity

Some bacteria such as *Salmonella typhi*, *styphylococcus aureus* and *E. coli* are used to determine the antiseptic value (the degree of antiseptic activity e.g. phenol co-efficient of certain drugs). The activity of antibiotics is also determined by using *Klebsiella pneumonia*, *Micrococcus flavus*, *Sarcira lutea* etc. living bacteria, yeast and molds are used to evaluate certain vitamins. Microbiological assays by cylinder plate method and turbidimetric method are used in evaluation.

3.5.2 Antifertility activity

Antifertility drugs include contraceptives and abortificients. Contraceptive drugs are used to prevent pregnancy and abortificient to terminate pregnancy. Female rats are used for

antifertility activity i.e. measure the pregnancy rate (antiovulation and anti-implantation) and male rats are used for antispermatogenic activity (inhibition of spermatogenesis) and spermicidal activity (sperm motility) of herbal drugs.

3.5.3 Hypoglycemic activity

Rabbits, rats or mice are used to test hypoglycemic activity of plant extract. Radio-immuno assay (RIA) or Enzyme linked immunosorbate assay (ELISA) are done for measurement of insulin levels.

3.5.4 Neuropharmacological activity

Testing the herbal drugs with effects on central and autonomic nervous system. CNS acting drugs like cocaine (*Erythroxylum coca*), morphine (*Papaver somniferum*), cannabinol (*Cannabis sativa*) are tested using rodents. For testing the herbal drugs for their effects on ANS guinea pig ileum for antispasmodic activity, rabbit jejunum for adrenergic activity, rat phrenic-nerve-diaphragm for muscle relaxant activity, frog rectus for skeletal muscles activity.

4. Analytical evaluation

In general, quality control is based on three important pharmacopoeias definitions:

Identity: Is the herb the one it should be?
Purity: Are there contaminants, e.g., in the form of other herbs which should not be there?
Content or assay: Is the content of active constituents within the defined limits.

It is obvious that the content is the most difficult one to assess, since in most herbal drugs the active constituents are unknown. Sometimes markers can be used which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not. To prove identity and purity, criteria such as type of preparation sensory properties, physical constants, adulteration, contaminants, moisture, ash content and solvent residues have to be checked. The correct identity of the crude herbal material, or the botanical quality, is of prime importance in establishing the quality control of herbal drugs [EMEA, 1998; Sharma, 1995; WHO, 1992].

Identity can be achieved by macro- and microscopical examinations. Voucher specimens are reliable reference sources. Outbreaks of diseases among plants may result in changes to the physical appearance of the plant and lead to incorrect identification.

Purity is closely linked with the safe use of drugs and deals with factors such ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. However, due to the application of improved analytical methods, modern purity evaluation includes microbial contamination, aflatoxins, radioactivity, and pesticide residues. Analytical methods such as photometric analysis (UV, IR, MS, and NMR), thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) can be employed in order to establish the constant composition of herbal preparations.

Content or assay is the most difficult area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes markers can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the

percentage extractable matter with a solvent may be used as a form of assay, an approach often seen in pharmacopeias. The choice of the extracting solvent depends on the nature of the compounds involved, and might be deduced from the traditional uses.

A special form of assay is the determination of essential oils by steam distillation. When the active constituents (e.g. sennosides in Senna) or markers (e.g. alkydamides in Echinacea) are known, a vast array of modern chemical analytical methods such as ultraviolet/visible spectroscopy (UV/VIS), TLC, HPLC, GC, mass spectrometry (MS), or a combination of GC and MS (GC/MS), can be employed [Booksh, 1994].

5. Chromatography and chemical fingerprints of herbal medicines

Several problems influence the quality of herbal drugs:

- Herbal drugs are usually mixtures of many constituents.
- The active principle(s) is (are), in most cases unknown.
- Selective analytical methods or reference compounds may not be available commercially.
- Plant materials are chemically and naturally variable.
- Chemo-varieties and chemo cultivars exist.
- The source and quality of the raw material are variable.
- The methods of harvesting, drying, storage, transportation, and processing (for example, mode of extraction and polarity of the extracting solvent, instability of constituents, etc.) have an effect.

Strict guidelines have to be followed for the successful production of a quality herbal drug. Among them are proper botanical identification, phytochemical screening, and standardization. Quality control and the standardization of herbal medicines involve several steps. The source and quality of raw materials, good agricultural practices and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in guaranteeing the quality and stability of herbal preparations [Blumenthal, 1998; EMEA, 2002; Roberts, 1997; WHO, 1992,1998, 2000, 2005, 2004].

The chemical constituents in component herbs in the herbal products may vary depending on stage of collection, parts of the plant collected, harvest seasons, plant origins (regional status), drying processes and other factors. Thus, it seems to be necessary to determine most of the phytochemical constituents of herbal products in order to ensure the reliability and repeatability of pharmacological and clinical research, to understand their bioactivities and possible side effects of active compounds and to enhance product quality control. Thus, several chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and thin layer chromatography (TLC), can be applied as quality assessment parameters. The concept of phytoequivalence was developed in Germany in order to ensure consistency of herbal products. According to this concept, a chemical profile, such as a chromatographic fingerprint, for an herbal product should be constructed and compared with the profile of a clinically proven reference product.

By definition, a chromatographic fingerprint of an herbal drug is, in practice, a chromatographic pattern of the extract of some common chemical components of

pharmacologically active and/or chemically characteristics. This chromatographic profile should be featured by the fundamental attributions of "integrity" and "fuzziness" or "sameness" and "differences" so as to chemically represent the herbal drug investigated. It is suggested that with the help of chromatographic fingerprints obtained, the authentication and identification of herbal medicines can be accurately conducted ("integrity") even if the amount and/or concentration of the chemically characteristic constituents are not exactly the same for different samples of drug (hence, "fuzziness") or, the chromatographic fingerprints could demonstrate both the "sameness" and "differences" between various samples successfully. Thus, we should globally consider multiple constituents in the herbal drug extracts, and not individually consider only one and/or two marker components for evaluating the quality of the herbal products. However, in any herbal drug and its extract, there are hundreds of unknown components and many of them are in low amount. Moreover, there usually exists variability within the same herbal materials. Consequently, to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components is not an easy or trivial work. Fortunately, chromatography offers very powerful separation ability, such that the complex chemical components in herbal extracts can be separated into many relatively simple sub-fractions [Ahirwal, 2006; Brain, 1975; Cheng, 2003; Clarke, 1967; Wanger, 1984].

In general, the methods for quality control of herbal medicines involve sensory inspection (macroscopic and microscopic examinations) and analytical inspection using instrumental techniques such as thin layer chromatography (TLC), HPLC, GC–MS, LC–MS, near infrared (NIR), and spectrophotometer, etc. On the other hand, the methods of extraction and sample preparation are also of great importance in preparing good fingerprints of herbal medicines. As a single herbal medicine may contain a great many natural constituents, and a combination of several herbs might give rise to interactions with hundreds of natural constituents during the preparation of extracts, the fingerprints produced by the chromatographic instruments, which may present a relatively good integral representation of various chemical components of herbal medicines [Bilia, 2002; Choi, 2002; Chuang, 1995; Ebel, 1987; Rozylo, 2002].

5.1 Thin layer chromatography

TLC was the most common, versatile method of choice for herbal analysis before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias such as Indian herbal pharmacopoeia, Ayurvedic pharmacopoeia; American Herbal Pharmacopoeia (AHP), Chinese drug monographs and analysis, Pharmacopoeia of the People's Republic of China, etc. Rather, TLC is used as an easier method of initial screening with a semi quantitative evaluation together with other chromatographic techniques as there is relatively less change in the simple TLC separation of herbal medicines than with instrumental chromatography.

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition or a combination of partition and

adsorption, depending on the particular type of support, its preparation and its use with different solvent [Herborne ,1928; Stahl, 1969].

Identification can be effected by observation of spots of identical Rf value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation.

TLC has the advantages of many-fold possibilities of detection in analyzing herbal medicines. In addition, TLC is rather simple and can be employed for multiple sample analysis. For each plate, more than 30 spots of samples can be studied simultaneously in one time. Thus, the use of TLC to analyze the herbal medicines is still popular. HPTLC is one of the sophisticated instrumental techniques based on the full capabilities of TLC. It is most flexible, reliable and cost efficient separation technique. The advantage of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hypenation, and so on enable it to be powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, food stuffs, and so on. With the help of the CAMAG video store system (CAMAG, Switerland) and TLCQA-UV methods, it is possible to get useful qualitative and quantitative information from the developed TLC plate. For example the four samples of *Cordyceps sinensis* from the joint products of China and Japan cooperation have more valuable medical effect compared to others as they contained the most effective component cordycepin. Moreover, with the help of image analysis and digitized technique developed in computer science, the evaluation of similarity between different samples is also possible.

The advantages of using TLC/HPTLC to construct the fingerprints of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation. Thus, TLC is a convenient method of determining the quality and possible adulteration of herbal products. It is worth noting that the new techniques of TLC are also being updated like forced-flow planar chromatography (FFPC), rotation planar chromatography (RPC), over pressured-layer chromatography (OPLC), and electro planar chromatography (EPC). A simple, but powerful preparative forced-flow technique was also reported; in this technique hydrostatic pressure is used to increase mobile-phase velocity. Parallel and serially-coupled layers open up new vistas for the analysis of a large number of samples (up to 216) for high throughput screening and for the analysis of very complex matrices [Funk, 1991;Gong, 2003; Svendsen, 1989; Wanger, 1996].

5.2 Gas chromatography

Gas chromatography (GC), also known as gas liquid chromatography (GLC), is a technique for separation of mixtures into components by a process which depends on the redistribution of the components between a stationary phase or support material in the form of a liquid, solid or combination of both and a gaseous mobile phase.

It is well-known that many pharmacologically active components in herbal medicines are volatile chemical compounds. Thus, the analysis of volatile compounds by gas chromatography is very important in the analysis of herbal medicines. The GC analysis of the volatile oils has a number of advantages. Firstly, the GC of the volatile oil gives a reasonable “fingerprint” which can be used to identify the plant. The composition and

relative concentration of the organic compounds in the volatile oil are characteristic of the particular plant and the presence of impurities in the volatile oil can be readily detected. Secondly, the extraction of the volatile oil is relatively straightforward and can be standardized and the components can be readily identified using GC-MS analysis. The relative quantities of the components can be used to monitor or assess certain characteristics of the herbal medicines. Changes in composition of the volatile oil may also be used as indicators of oxidation, enzymatic changes or microbial fermentation. The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds. This is especially true for the usual FID detection and GC–MS. Furthermore, the high selectivity of capillary columns enables separation of many volatile compounds simultaneously within comparatively short times. Thus, over the past decades, GC is a popular and useful analytical tool in the research field of herbal medicines. Especially, with the use of hyphenated GC–MS instrument, reliable information on the identity of the compounds is available as well. However, the most serious disadvantage of GC is that it is not convenient for its analysis of the samples of polar and non-volatile compounds. For this, it is necessary to use tedious sample work-up which may include derivatization. Therefore, the liquid chromatography becomes another necessary tool for us to apply the comprehensive analysis of the herbal medicines [Nyiredy, 2003].

The first fully automated on-line GC-IR system was developed by Scott et al. Each eluted solute was adsorbed in a cooled packed tube, and then thermally regenerated into an infrared vapor cell. Subsequent to the IR spectrum being obtained, a small sample of the vapor was drawn from the IR cell into a low-resolution mass spectrometer and the mass spectrum was also taken [Gong, 2001; Yan, 2009; Ylinen, 1986].

5.3 High-performance liquid chromatography

High performance liquid chromatography (HPLC), also known as high pressure liquid chromatography, is essentially a form of column chromatography in which the stationary phase consists of small particle (3-50μm) packing contained in a column with a small bore (2-5mm), one end of which is attached to a source of pressurized liquid eluent (mobile phase). The three forms of high performance liquid chromatography most often used are ion exchange, partition and adsorption.

HPLC is a popular method for the analysis of herbal medicines because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. Thus, over the past decades, HPLC has received the most extensive application in the analysis of herbal medicines. Reversed-phase (RP) columns may be the most popular columns used in the analytical separation of herbal medicines.

It is necessary to notice that the optimal separation condition for the HPLC involves many factors, such as the different compositions of the mobile phases, their pH adjustment, pump pressures, etc. Thus, a good experimental design for the optimal separation seems in general necessary. In order to obtain better separation, some new techniques have been recently developed in research field of liquid chromatography. These are micellar electrokinetic capillary chromatography (MECC), high-speed counter-current chromatography (HSCCC), low-pressure size-exclusion chromatography (SEC), reversed-phase ion-pairing HPLC (RP-

IPC-HPLC), and strong anion-exchange HPLC (SAX-HPLC). They will provide new opportunities for good separation for some specific extracts of some herbal medicines. On the other hand, the advantages of HPLC lie in its versatility for the analysis of the chemical compounds in herbal medicines, however, the commonly used detector in HPLC, say single wavelength UV detector, seems to be unable to fulfill the task, since lots of chemical compounds in herbal medicines are non-chromophoric compounds. Consequently, a marked increase in the use of HPLC analysis coupled with evaporative light scattering detection (ELSD) in a recent decade demonstrated that ELSD is an excellent detection method for the analysis of non-chromophoric compounds. This new detector provides a possibility for the direct HPLC analysis of many pharmacologically active components in herbal medicines, since the response of ELSD depends only on the size, shape, and number of eluate particles rather than the analysis structure and/or chromophore of analytes as UV detector do. Especially, this technique is quite suitable for the construction of the fingerprints of the herbal medicines. Moreover, the qualitative analysis or structure elucidation of the chemical components in herbal drug by simple HPLC is not possible, as they rely on the application of techniques using hyphenated HPLC, such as HPLC-IR, HPLC–MS, HPLC-NMR, for the analysis of herbal medicines [Lazarowych, 1998; Li, 1999; Liu, 1999; Li, 2003; Tsai, 2002; Liu, 1993; Zhang, 2004].

5.4 Electrophoretic methods

Capillary electrophoresis was introduced in early 1980s as a powerful analytical and separation technique and has since been developed almost explosively. It allows an efficient way to document the purity/complexity of a sample and can handle virtually every kind of charged sample components ranging from simple inorganic ions to DNA. Thus, there was an obvious increase of electrophoretic methods, especially capillary electrophoresis, used in the analysis of herbal medicines in last decades. The more or less explosive development of capillary electrophoresis since its introduction has to a great extent paralleled that of liquid chromatography. Most of the used techniques are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and capillary isoelectric focusing (CIEF). CE is promising for the separation and analysis of active ingredients in herbal medicines, since it needs only small amounts of standards and can analyze samples rapidly with very good separation ability. Also, it is a good tool for producing the chemical fingerprints of the herbal medicines, since it has similar technical characteristics of liquid chromatography. Recently, several studies dealing with herbal medicines have been reported and two kinds of medicinal compounds, i.e. alkaloids and flavonoids, have been studied extensively.

In general, CE is a versatile and powerful separation tool with high separation efficiency and selectivity when analyzing mixtures of low-molecular-mass components However, the fast development in capillary electrophoresis causes improvement of resolution and throughput rather than reproducibility and absolute precision. One successful approach to improve the reproducibility of both mobility and integral data has been based on internal standards. But many papers were published unfortunately revealed limited image on the real possibilities of CE in the field of fingerprinting herbal medicines. CE and capillary electrochromatography approaches contribute to be a better understanding of the solution behavior of herbal medicines, especially when additionally combined with the powerful spectrometric detectors [Liu, 1992, 1993; Stuppner ,1992; Yang, 1995].

6. Hyphenation procedures

In the past two decades, combining a chromatographic separation system on-line with a spectroscopic detector in order to obtain structural information on the analytes present in a sample has become the most important approach for the identification and/or confirmation of the identity of target and unknown chemical compounds. For most (trace-level) analytical problems in the research field of herbal medicines, the combination of column liquid chromatography or capillary gas chromatography with a UV-VIS or a mass spectrometer (HPLC-DAD, CE-DAD, GC-MS and LC-MS, respectively) becomes the preferred approach for the analysis of herbal medicines.

The additional and/or complementary information required in number of cases can be provided by, for example, atomic emission, Fourier-transform infrared (FTIR), fluorescence emission (FE), or nuclear magnetic resonance (NMR) spectrometry. It is demonstrated that, from a practical point of view, rewarding results can be obtained, since we need much more information to deal with the most complex analytical systems such as those samples from herbal medicines. Furthermore, the data obtained from such hyphenated instruments are the so-called two-way data; say one way for chromatogram and the other way for spectrum, which could provide much more information than the classic one-way chromatography. With the help of chemo metrics, a rather new discipline developed both in chemistry and statistics in the later part of the 1970s, we will definitely get more chance to deal with the difficult problems in the analysis of herbal medicines and also the problems in quality control of herbal medicines.

6.1 LC-IR, LC-MS, LC-NMR

The hyphenated technique developed from the coupling of liquid chromatography and infrared spectroscopy is known as LC-IR. LC-IR is an important technique as it shows absorption peaks of functional groups in mid IR region which helps in structural identification of compounds present in a sample. The detection technique of IR is comparatively slow than other techniques like MS or NMR. Two approaches used in these techniques are flow cell approach and solvent elimination approach.

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the specific detection and potential identification of chemicals in a complex mixture.

There are two common atmospheric pressure ionization (API) LC/MS process: Electrospray Ionization (ESI) & Atmospheric Pressure Chemical Ionization (APCI). Both are soft ionization technique. Both of these processes are compatible with most chromatographic separations.

The combination of liquid chromatography (LC) and nuclear magnetic resonance (NMR) offers the potential of unparalleled chemical information from analytes separated from complex mixtures. Several other hyphenated NMR techniques have been developed to enhance sensitivity of this technique. LC-SPE-NMR increases sensitivity of the instrument by utilizing a solid phase extraction device after LC column. Capillary LC-NMR also practically lowers detection limit to a nanogram range through integration of capillary LC

with NMR detection. Further Cryo-LC-probe technology combine the advantage of sample flow and enhanced sensitivity from a cryogenically cooled NMR probe [Wang, 2001].

6.2 GC-MS

Mass spectrometry is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. Combining chromatography with mass spectrometry provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method. In mass spectrometry, there is a range of methods to ionize compounds and then separate the ions. Common methods of ionization used in conjunction with gas chromatography are electron impact (EI) and electron capture ionization (ECI). EI is primarily configured to select positive ions, whereas ECI is usually configured for negative ions (ECNI). EI is particularly useful for routine analysis and provides reproducible mass spectra with structural information which allows library searching. GC–MS was the first successful online combination of chromatography with mass spectrometry, and is widely used in the analysis of essential oil in herbal medicines.

With the GC–MS, not only a chromatographic fingerprint of the essential oil of the herbal medicine can be obtained but also the information related to its most qualitative and relative quantitative composition. Used in the analysis of the herbal medicines, there are at least two significant advantages for GC–MS, that is: (1) with the capillary column, GC–MS has in general very good separation ability, which can produce a chemical fingerprint of high quality; (2) with the coupled mass spectroscopy and the corresponding mass spectral database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC–MS, which will be extremely useful for the further research for elucidating the relationship between chemical constituents in herbal medicine and its pharmacology in further research. Thus, GC–MS should be the most preferable tool for the analysis of the volatile chemical compounds in herbal medicines [Gong, 2001, 2003; Li, 1999, 2003,].

6.3 HPLC–DAD, HPLC–MS and others

HPLC–DAD has become a common technique in most analytical laboratories in the world now. With the additional UV spectral information, the qualitative analysis of complex samples in herbal medicines turns out to be much easier than before. For instance, checking peak purity and comparing with the available standard spectrum of the known compound to the one in the investigated sample. Especially, with the introduction of electrospray mass spectrometry, the coupling of liquid chromatography and mass spectrometry has opened the new way to widely and routinely applied to the analysis of herbal medicines. HPLC chromatographic fingerprints can be then applied for documentation of complete herbal extracts with more information and on-line qualitative analysis becomes possible. Several valuable review articles dealing with LC–MS and its application in the analysis of botanical extracts have been published, In last decades, the increasing usage of LC–MS and HPLC–DAD in the analysis of herbal medicines is quite obvious. Several good reviews have been published for the analysis of the bioactive chemical compounds in plants and herbal medicines, in which the technique used most is HPLC, especially the hyphenated

HPLC techniques. Moreover, combined HPLC–DAD–MS techniques take advantage of chromatography as a separation method and both DAD and MS as an identification method. DAD and MS can provide on-line UV and MS information for each individual peak in a chromatogram. With the help of this hyphenation, in most cases, one could identify the chromatographic peaks directly on-line by comparison with literature data or with standard compounds, which made the LC–DAD–MS becomes a powerful approach for the rapid identification of phytochemical constituents in botanical extracts, and it can be used to avoid the time-consuming isolation of all compounds to be identified. Recently, the hyphenation between HPLC and NMR are also available, which might become a vital and an attractive analytical tool for the analysis of drugs in biological fluids and for the analysis of herbal medicines. In fact the tendency of the hyphenation or multi-hyphenation of the chromatography with the common used four spectroscopic detectors, say UV, Fourier transformation infrared spectrum, MS and NMR, for structure elucidation of chemical compounds, is in progress. A "total analysis device" has been recently demonstrated in the case of on-line HPLC–UV (DAD)–FT–IR–NMR–MS analyses. It may be worth noting that the electrode array hyphenated with HPLC is also in progress. As electrochemical detection was described to be superior to UV–Vis and fluorescence spectroscopy for determination of some chemical compound, like polyphenols in trace levels, very sensitive determination was achieved by using a multi channel electrochemical detector (Coul Array). A two-way chromatogram is obtained, since the detector has 12 (or more) electrodes in series set incrementally to different potentials. Thus, similar to the UV–Vis spectra, the hydrodynamic voltammogram can be used for peak purity checking and peak identification [Maillard, 1993; Mellon, 1987; Rajani, 2001; Ravilla, 2001; Sticher, 1992; Wolfender, 1993, 1994, 1995].

6.4 Hyphenation of CE

The situation of the CE analysis in hyphenation development is somewhat like HPLC analysis. The hyphenated CE instruments, such as CE-DAD, CE-MS and CE-NMR, all appeared in the past decades. The techniques have also quickly been used for the analysis of the samples from herbal medicines. On-line coupling of capillary electrophoresis to mass spectrometry and other spectrometry allows both the efficient separation of CE and the specific and sensitive detection to be achieved. Furthermore, the artifacts happened in CE measurements might be overcome with the help of some information handling technique, such as some methods developed in chemometrics, since one could use with the additional information from spectra to correct the artifacts from the chosen separation buffer chemistry or from hidden instrumental constraints. In sum, as the hyphenated techniques in chromatographic and electrophoretic instruments develop, our ability of analysis of herbal medicines, both in qualitative and quantitative respects and our ability of quality control of herbal medicines will become stronger and stronger. We are quite sure that we will have a very prospective future for quality control of herbal medicines [Pusecker, 1998; Stockigt, 2002; Schewitz, 1998].

CE analysis can be driven by electric field performed in narrow tubes which can result in rapid separation of hundreds of compounds. It separates components by applying voltage in between buffer filled capillaries. The components are separated due to production of ions

depending on their mass and charge. It is widely used in quantitative determination and the analysis particularly the assay development and trace level determination. When MS is linked to CE then it produces determination of molecular weight of components often termed as CE-MS. Separation is achieved from the etched surface of the capillaries that delivers sample to the ESI MS. This technique runs in full automation and having higher sensitivity and selectivity. The new interface known as coaxial sheath interface has developed which has potential of use of both CE-MS and LC-MS alternatively on same mass spectrometer.

Information features of chromatographic fingerprints of herbal medicines

Hence there are many chromatographic techniques, including the hyphenated chromatographies, available for us to do the instrumental analysis of herbal medicines, and to construct further their fingerprinting. The problem here is that how could we efficiently and reasonably evaluate such-obtained analytical results and/or the fingerprints of the herbal medicines and how could we use the information obtained from chromatographic analysis to address further the problem of quality control for herbal medicines [Liu, 1994, 1993].

7. Phytoequivalence and chromatographic fingerprints of herbal medicines

In general, one could use the chromatographic techniques to obtain a relatively complete picture of an herbal, which is in common called chromatographic fingerprints of herbal medicines to represent the so-called phytoequivalence. The following results show some examples. Fig. 1 shows the total ionic chromatograms (TIC) of essential oils in *Cortex cinnamomi* from four producing areas, say: (a) Zhaoqing, Guangdong province, China; (b) Yulin, Guangxi province, China; (c) Yunnan province, China; and (d) Vietnam, by GC–MS. There are, of course, some differences in the profiles. However, seen from these profiles, phytoequivalence is obvious for *C. cinnamomi*. The other example is shown in Fig. 2a Superimposed HPLC-UV chromatogram of *Ginkgo biloba* in commercial products, Fig 2b in which there are chromatograms of methanol extracts of *Erigeron breviscapus* from 32 different producing places in the same province by HPLC detected at wavelength 280 nm. The integrated feature of the chromatographic fingerprints of herbal medicines can be clearly seen from these examples.

Obtaining a good chromatographic fingerprint representing the phytoequivalence of a herb depends several factors, such as the extracting methods, measurement instruments, measurement conditions, etc. In fact, if we want to obtain an informative fingerprint of a herbal medicine, we need to have a good extracting method, with which we could fortunately obtain almost all the pharmaceutically active compounds to represent the integrity of the herbal medicine.

Furthermore, a chromatogram with good separation and a representative concentration profile of the bioactive components detected by a proper detector are also required. Thus, how to obtain a high quality chromatographic fingerprint of as more as possible information of the herbal medicines is an important task for chemists and pharmacologists. In order to understand bioactivities and possible side effects of active compounds of the herbal

medicines and to enhance product quality control, it seems that one needs to determine most of the phytochemical constituents of herbal products so as to ensure the reliability and repeatability of pharmacological and clinical research. Suppose that we have obtained some fingerprints, how to evaluate the information contents of the chromatographic fingerprints of herbal medicines reasonably and efficiently is the second step for the quality control purpose [Beek, 2002; Caoa, 2006; Gong, 2003; Hasler, 1992; Kinghorn, 1998; Liang, 1994; Upton, 2001].

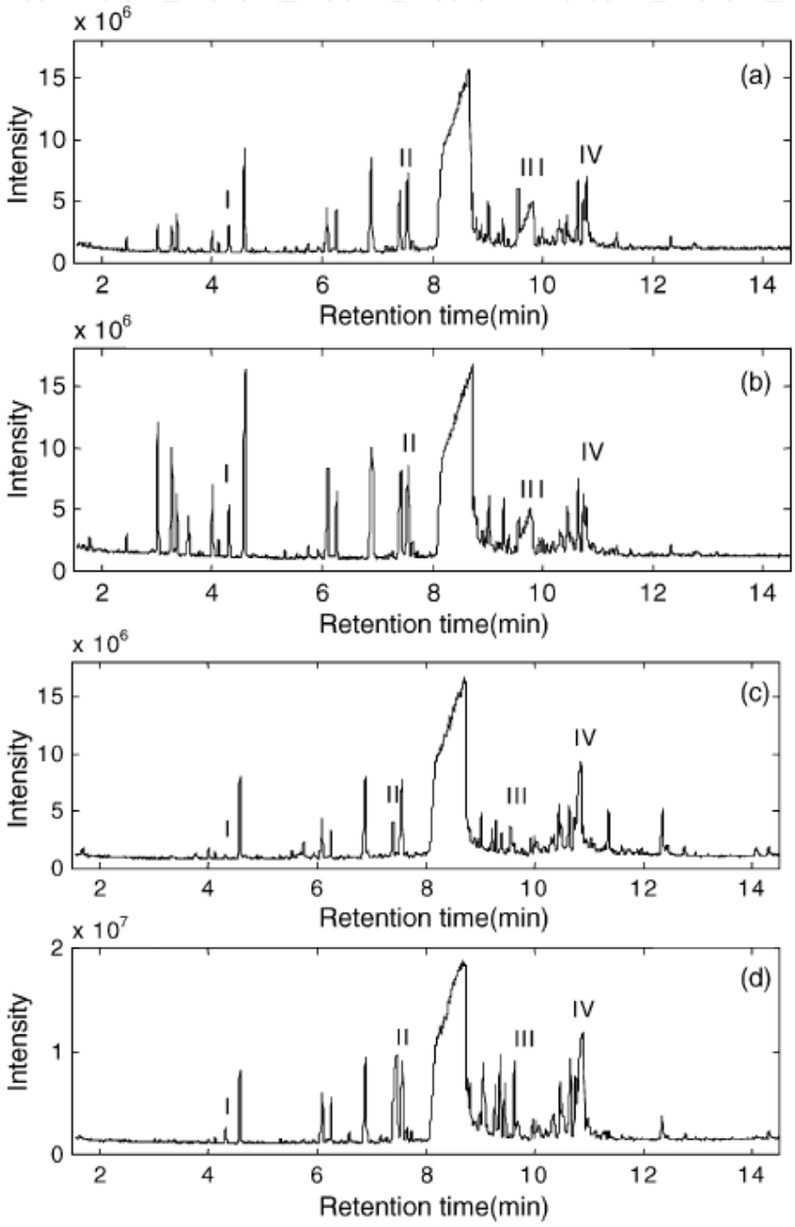


Fig. 1. Total ionic chromatograms of essential oils in *C. cinnamomi* from four different producing areas: (a) Zhaoquing, Guangdong province, china; (b) Yulin, Guangxi province, china; (c) Yunnan province, china; and (d) Vietnam.

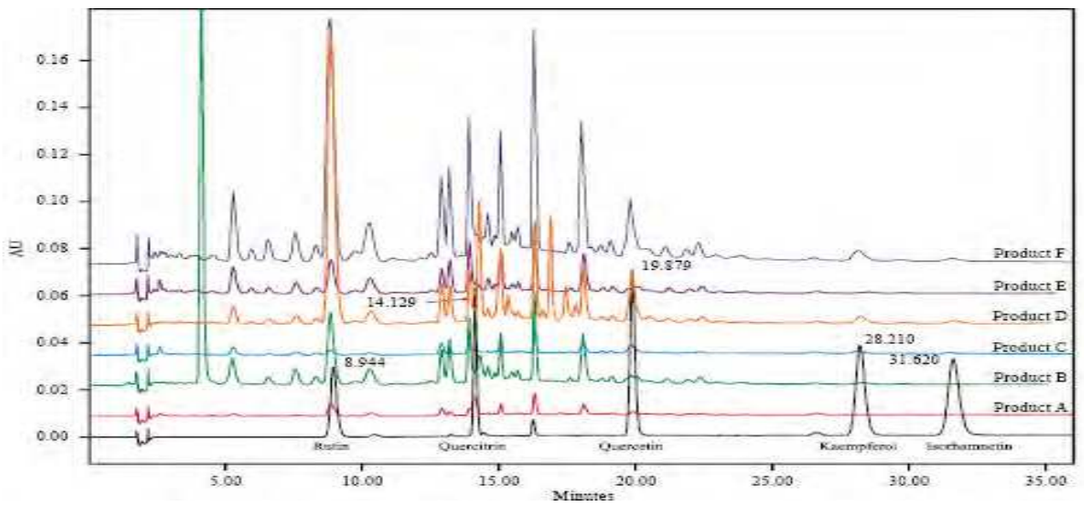


Fig. 2a. Superimposed HPLC-UV chromatogram of *Ginkgo biloba* in commercial products.

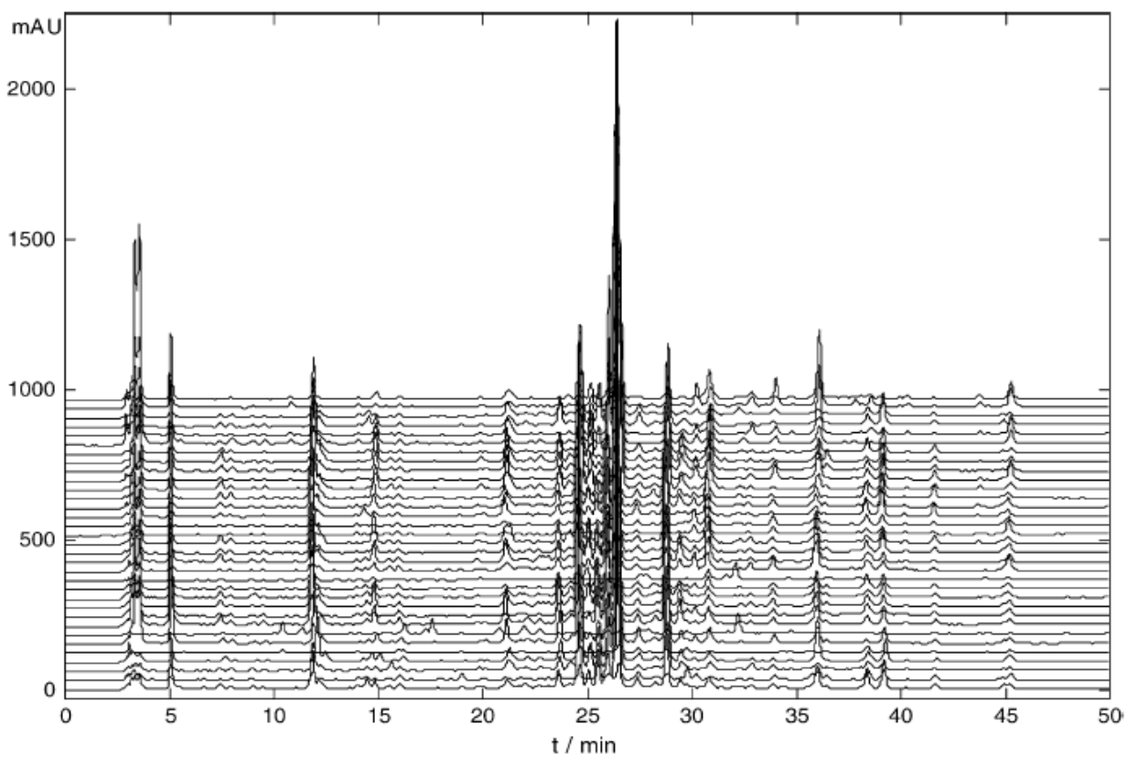


Fig. 2b. Original HPLC chromatograms of 33 herbal samples of *Erigeron breviscapus* at wavelength λ=280nm.

7.1 Information contents of fingerprints of herbal medicines

It is obvious that a chromatographic fingerprint of an herbal medicine is a multivariate system, since in general it embraces most of the phytochemical constituents of herbal

products. From a point of view of multivariate, the information content of a chromatogram with lots of peaks might be calculated by means of various approaches. However, as for these methods, the signal intensity, retention time, peak area and/or peak height of each independent peak without overlapping should be all taken into consideration; the calculation burden is also rather heavy. Moreover, if a chromatographic peak is overlapped with its adjacent peak(s), the calculation of the information content will become complex. Vertical splitting is conventionally used for this situation and both peaks on two sides of an overlapping peak cluster are taken as pure ones. Out of question, this approximate treatment on a chromatogram with some overlapping peak clusters will cause some errors on calculation of information content.

In fact, a chromatographic fingerprint, which is a concentration distribution curve of several chromatographic peaks, could be regarded as a continuous signal determined by its chromatographic shape. According to information theory, the information content of a continuous signal might be simply expressed as the following formula:

$$\Phi = -\sum pi \log pi \tag{1}$$

where pi is the positive real numbers of probability property, say $\sum pi = 1$. Based on this idea, we proposed recently a simple method to calculate the information content (Φ) for chromatographic fingerprints of herbal medicines as shown in the following equation, that is

$$\Phi = -\sum \left(\frac{xi}{\sum xi} \log\left(\frac{xi}{\sum xi}\right)\right) \tag{2}$$

where xi is the real chromatographic response of the chemical components involved in the chromatographic fingerprint under study. Here, the normalization of xi divided by their sum is to make the chromatogram investigated be of probability property. In theory, if and only if xi with unchangeable variance is characterized by normal distribution can its information content Φ reach its maximum.

Under an ideal situation, all the chromatographic peaks from a chromatogram can be separated completely and each peak confined to a narrow zone might correspond to a normal distribution profile. A chromatographic fingerprint with all of peaks just completely separated should be featured by maximal information content. Further separation cannot provide any more information and becomes unnecessary. On the contrary, if any of chromatographic peaks is overlapped with its adjacent one(s), this peak will surely show non-Gaussian normal distribution and therefore undoubtedly cause a loss of the information content. There might be, at least, two advantages of the calculation of the information content of complex chromatograms over the available approaches. First, the method uses the whole chromatogram with a simple normalization, thus it is not necessary to identify the retention time, peak intensity, peak width, peak area and/or peak height for all the peaks identified. The calculation burden is reduced significantly. Moreover, the theoretic background of the method is simple and reasonable [Gong, 2003; Hayashi, 1990; Huber, 1993; Matsuda, 1989].

7.2 Correction of retention time shift of fingerprints of herbal medicines

When one deals with several chromatographic fingerprints obtained from the same herbal medicine or from different sources, the first step of our task might be to evaluate their similarity and difference between them. However, correction of retention time shift of the fingerprint of herbal medicines should be taken into consideration first, since some types of variation sources are inevitably encountered from one chromatogram to another. Under this situation, some unacceptable results, one of which is imposed by the retention time shifts, will be produced. Unfortunately, how the retention time shifts are caused by the variation sources is very complex. It might be due to (1) the degradation of the stationary phase, especially, the low stability of silica and silica-based supports at high pH values and the collapse of C- 18 bonded phase because of a highly polar mobile phase; (2) minor changes in mobile phase composition caused by temperature and pressure fluctuations, variations in flow-rate and gradient dispersion; (3) some problems involved in the detectors, for example, a wavelength shift in the UV spectrometer, a spectroscopic intensity variation and the misalignment of the monochromator; (4) the column overloading on account of the great injected amount or some components with high concentration; (5) the possible interaction between analytes; (6) other unknown shifts in the instrument. If these disadvantageous cases are in existence during the chromatographic runs, the retention times will be subsequently shifted. When such retention time shifts occur in chromatographic fingerprints, it is great difficult to conduct data processing, for example, the construction of common chromatographic models of all samples investigated, the similarity comparison between chromatographic fingerprints and pattern recognition based on principal component analysis (PCA) since multivariate analysis with entire chromatographic profiles as input data is very sensitive to even minute variations. As a result, in order to make up a consistent data, it is necessary to detect the retention time shifts of chromatographic fingerprints and then the chromatographic profiles should be adjusted along the retention time direction by means of synchronizing the retention times of the chromatographic peaks from the same components. During the past decades, several kinds of useful approaches have been developed for peak synchronization in chromatographic profiles. Some of them corrected the retention time shifts by making internal standards added or marker peaks coincide in all chromatograms under study. At its first step, chromatographic peaks were identified by setting a retention time window in both of the sample and target chromatograms, and then a list of their retention times was generated. Here, the window meant the maximum shift to be considered. Clearly, the choice of the window size was critical to this technique. However, if the retention time shifts are very serious for complex systems like herbal medicines, the selection of the optimal peak-matching Windows might not be a trivial task. The objective functions on the correlation between the target and sample chromatograms were optimized and then the sample chromatographic profiles were aligned with the target [Bylund, 2002; Hamalainen, 1993; Hayashi, 1990; Huber, 1993; Matsuda, 1989; Pusecker, 1998; Schewitz, 1998].

In general, the methods must be very efficient and elegant if the samples investigated are quite similar in the concentration profiles of the chemical components. However, if the concentration profiles change greatly for the complex samples such as herbal medicines from the different producing places and/or from the various harvest seasons, wrong results might be obtained by simply seeking the optimal correlation coefficient between the chromatograms. It is because that the correlation coefficient is influenced greatly by the big

peaks in the chromatographic profiles. In this case, the maximal correlation coefficient does not certainly represent the best correction for the retention time shifts. However, if the data from the hyphenated chromatographic instruments, such as HPLC–DAD, GC–MS, etc. the correction of retention time shift of the fingerprint of herbal medicines will become much clear and easy, which we will discuss later on.

7.3 Evaluation of chemical fingerprints of herbal medicines

In the early chemometric research, chromatographic data were commonly first transformed to retention time–peak area data matrices including only selected peaks, whether the identity of the peaks are known or not. The data such obtained were then used to do the processing, that is, the calculation of similarity or dissimilarity between the fingerprints and the analysis of principal component analysis. However, as pointed by Nielsen, "The quality of the data (including only retention time–peak area data) relies on peak detection (integration) and on how the peaks are selected for the data analysis. It can be very difficult to select an optimal set integration parameters for chromatograms obtained from analysis of complex samples which easily contain more than 100 peaks. Furthermore, the selection and extraction of peaks to include in data analysis is difficult, partly subjective and large amounts of the data in the chromatograms are discarded. The disadvantages of peak detection and integration, and of the introduction of a subjective peak selection can be avoided by using all collected data points in the chemometric analysis." Thus, following the suggestion of Nielsen, the entire chromatographic profiles were utilized to perform direct chemometric analysis. The analysis can be easily done with the help of proper techniques of data compression, such as the technique of wavelet or Fourier transformation, if necessary. Furthermore, another advantage of taking the entire chromatographic profile to perform direct chemometric analysis is that the peak shape can be included in data analysis, which will make the pretreatment of overlapping peaks much easier when one does evaluation of the fingerprints. Of course, the chromatographic profiles should properly aligned to compensate for minor drifts in retention times before one does the fingerprint evaluation and chemometric analysis for the purpose of quality control [Martens, 1991; Sticher, 1992, 1993; Tauler, 1992; Wold, 1987; Zhang, 2003].

7.4 Fingerprints and quality control in herbal medicines

As stated above, one or two markers or pharmacologically active components were currently employed for evaluating the quality and authenticity of an herbal medicine. This kind of the determination, however, does not give a complete picture of an herbal product and therefore it will definitely fail to do the identification of false and true plant extraction. In the following an example will be given to illustrate the situation. Fig. 3 shows 17 fingerprints of *Ginkgo biloba* extractions, which were purchased from several pharmaceutical stores, vendors/companies and collected from various producing areas in the mainland of PR China. All of these samples were supposed to meet the standard measured by UV spectroscopy at wavelength of 318 nm with satisfactory absorbance (old standard method for quality control of *G. biloba* extraction in China), among which standard extract EGb761 from Guangzhou Institute for Drug Control, PR China by a Frenchman from Beaufour-Ipsen Company in France with a satisfactory fingerprint pattern. Analytical grade methanol and phosphoric acid used for mobile phase and all reagents were also of analytical grade. Ultrapure water (18.2M) was obtained by means of a Milli-Q apparatus by Millipore

Corporation (France) and was used for mobile phase preparation. The mobile phase was vacuum filtered through a filter of 0.45 μm pore pore size. From the plot, it is difficult to find some false one. But if we simply do the PCA upon the fingerprints, the results are shown in Fig. 4.

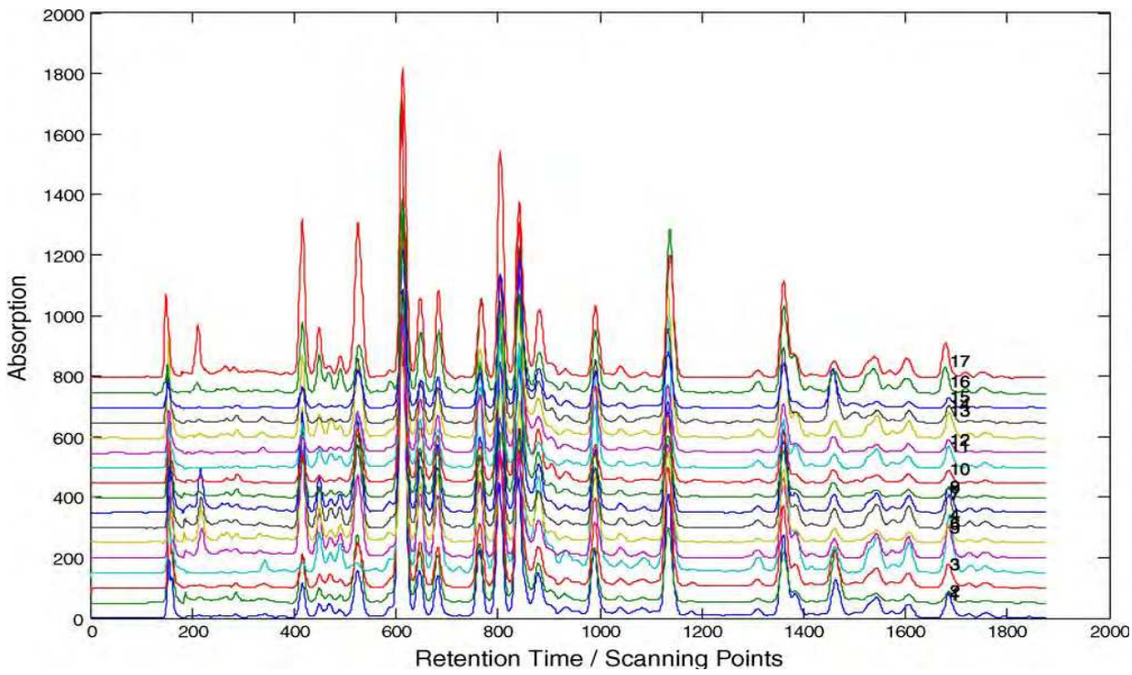


Fig. 3. Chromatograms of 17 extracts of *Ginkgo biloba* meet with the standard measured at wavelength 318nm.

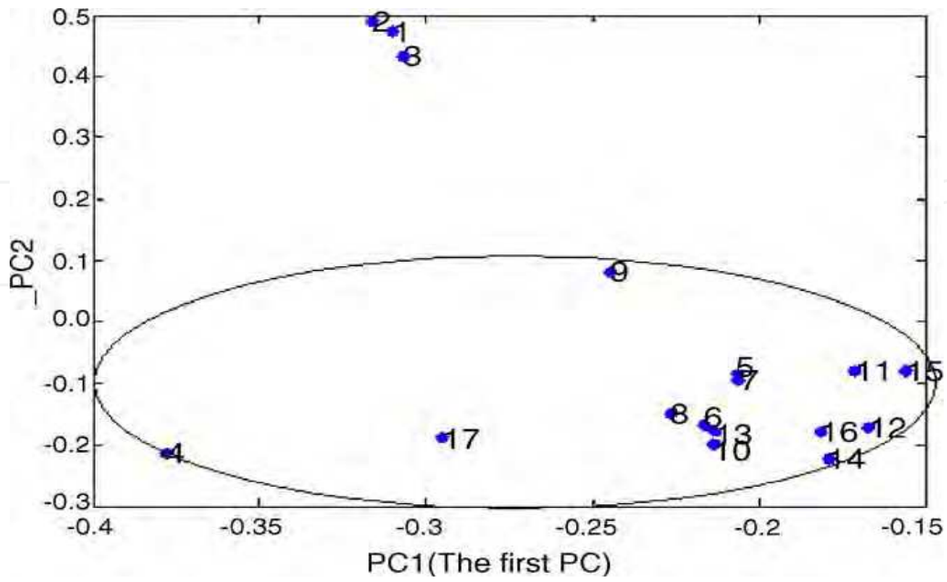


Fig. 4. The score plot obtained by principal components analysis where PC1 means the scores coordinates of principal component 1 and PC2 the ones of principal component 2.

It can be easily seen from the plot that samples marked by numbers 1–3 are clearly outliers. Thus, if we pick up the fingerprints of samples 2 and 3 (see Fig. 5C and D) and comparing them with the fingerprints (see Fig. 5A and B) of the standard extract EGb761 (number 17 in Fig. 4) and the other sample (number 8 in Fig. 4), we can easily find the difference between them. The peak in the fingerprints of samples 2 and 3 around the retention time of 10 min is much higher than the one in the standard extract EGb761 and sample 8. This peak is rutin. In fact, rutin was added in the three outlier samples, say samples 1–3, in order to meet the old standard of enough absorbance. They are quite different from the real *G. biloba* extractions as shown in Fig. 5A. From this example, we can see that the technique of fingerprint could really identify the false herbal products [Chau, 1996].

7.5 Similarity of fingerprints

The construction of chromatographic fingerprints aims at evaluating the quality of herbal medicines. As discussed above, the fundamental reason of quality control of herbal medicines is based on the concept of phytoequivalence of herbs, and then to use this conception to identify the real herbal medicine and the false one, and further to do quality control. Thus, the intuitive evaluation method is to compare the similarities and/or differences of the chromatographic fingerprints' shape. As a result, both the separation degrees and concentration distribution of components involved in a chromatographic fingerprint is also taken into consideration for this evaluation. The most commonly used standards for evaluation of similarity of the multivariate systems are correlation coefficient and congruence coefficient as expressed by the following two formulae correlation coefficient:

$$r_1 = \frac{\sum(xi-\bar{x})(yi-\bar{y})}{(\sum(xi-\bar{x})^2)^2(\sum(yi-\bar{y})^2)^2} \qquad (i= 1, 2,\ldots\ldots n) \tag{3}$$

congruence coefficient:

$$r_1 = \frac{\sum xiyi}{(\sum(xi^2))^{1/2}(\sum(yi)^2)^{1/2}} \qquad (i=1, 2,\ldots\ldots..n) \tag{4}$$

where xi, yi are the ith elements in two different fingerprints, say x and y, respectively and n is the number of the elements in the fingerprints. $\bar{x}$ and $\bar{y}$ are the mean values of the n elements in fingerprints x and y, respectively, that is,

$$\bar{x} = \frac{\sum xi}{n} \tag{5}$$

$$\bar{y} = \frac{\sum yi}{n} \tag{6}$$

The relationship within a set of chromatographic fingerprints could be currently analyzed through comparison in terms of similarity or dissimilarity of the objects with a certain reference, presented as correlation coefficient or congruence coefficient.

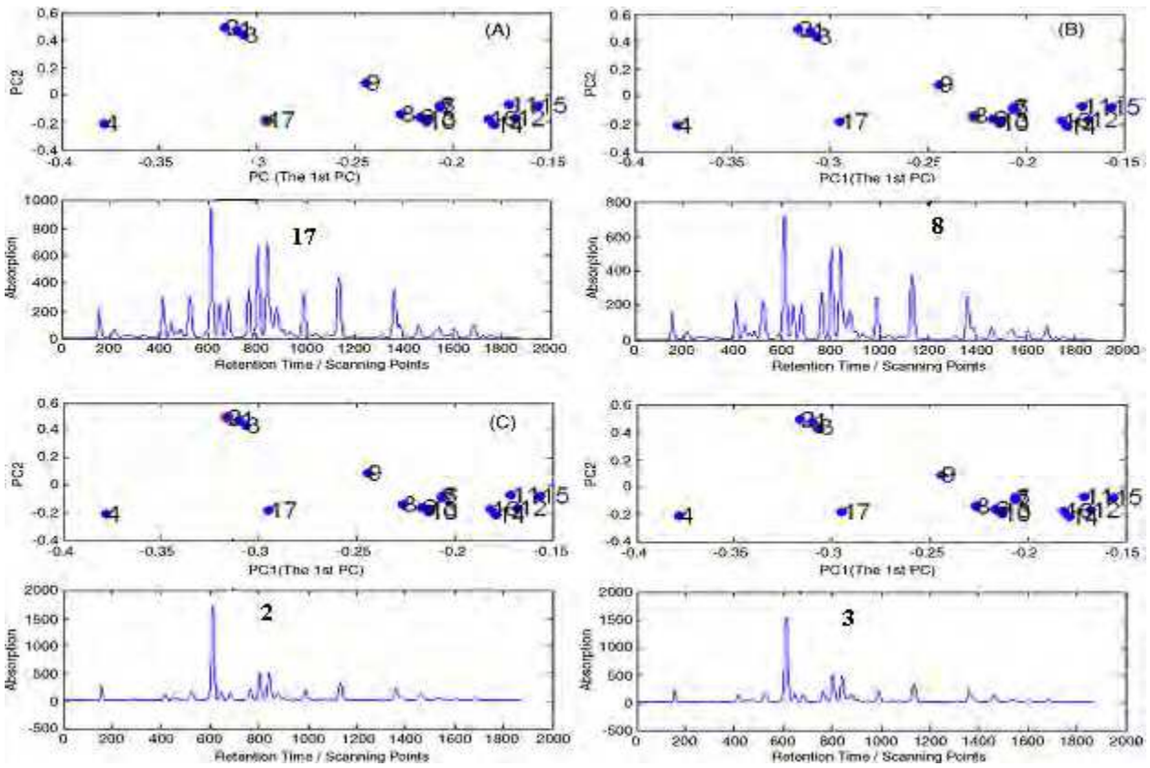


Fig. 5. The relationship between the projection points of score plot of PCA and the corresponding original fingerprints for samples 17, 8, 2 and 3, respectively.

In order to illustrate the situation, the example used in the above subsection is now applied to address such a problem. Table 1 list the correlation coefficients and congruence coefficients of the samples compared with the median spectrum of the whole 17 samples. From the table, we can see that the three false extractions have the smallest similarity values which seem to suggest that the similarity estimation may be used as a standard together with the original fingerprint, even this is very simple.

Sample no.	Correlation coefficients	Congruence coefficients
1	0.8795	0.8939
2	0.8795	0.8899
3	0.8935	0.9051
4	0.9349	0.9474
5	0.9511	0.9604
6	0.9469	0.9574
7	0.9552	0.9637
8	0.9779	0.9821
9	0.9524	0.9568
10	0.9158	0.9269
11	0.9869	0.9895

Sample no.	Correlation coefficients	Congruence coefficients
12	0.9506	0.9593
13	0.9753	0.9801
14	0.9479	0.9581
15	0.9590	0.9640
16	0.8979	0.9187
17	0.9436	0.9547

Table 1. Correlation coefficients and congruence coefficients of the samples compared with the median spectrum of the whole 17 samples.

7.6 Chemical pattern recognition and classification evaluation

As discussed above, the relationship within a set of chromatographic fingerprints could be currently analyzed through comparison in terms of similarity or dissimilarity of the objects with a certain reference, presented as correlation coefficient or congruence coefficient, etc. But, it has been aware that there are two problems for this comparison: how to achieve the reasonable reference (comparing standard) and to what extent the investigated object is similar with the reference. Popularly, the reference may be derived either from standard extract of herbal medicine or proportioned mixture of herbal medicine (e.g. EGb761) or from computation by some mathematical methods (for the example above, the median chromatographic fingerprint of whole samples is taken, since there are three outliers in the samples investigated). However, it is well known that natural products derived from herbal medicines with inherent "uncertainty" feature of its secondary metabolic substances, to define an absolute reference fingerprint by simply calculating their mean or median for one kind of herbal medicine seems somewhat subjective.

From this point of view, the conception of class of one herbal medicine seems to be more reasonable. Thus, the chemical pattern recognition methods, such as K-nearest neighbors (KNN) and soft independent modeling of class analogy (SIMCA), etc. should be taken into consideration for reasonable definition of the class of the herbal medicine. In fact, several researchers in China had worked on the concepts of using chemical analytical and chromatographical fingerprinting to measure the consistency of raw Chinese medicinal herbs and composite formula with the application of fuzzy clustering analysis of HPLC pattern in the early 1990s.

On the other hand, the (dis)similarities of herbal objects with the reference often undertake themselves to a qualified threshold, which is not so easy to define. Although such a comparison attaches importance to the integral relationship of the fingerprints, sometimes masking and swamping effects might occur either explicitly or implicitly. The masking effect is that an unexpected sample is undecided because of high similar value (e.g. the identification of three species of *Coptis chinensis*, *C. teet-Oides C. Y. Cheng*, and *C. deltoidea C. Y. Cheng et Hsiao* from herb *Rhizoma coptidis*). The swamping effect encompasses wrongly discriminating a desirable sample illegal on account of low similarity with the reference influenced by the diversity of chromatographic compositional distribution (e.g. the determination of herb *Houttuynia cordata* Thunb. from different sources). To avoid these effects as much as possible, a method based on PCA after necessary data transformations.

The method has been demonstrated that PCA with standard normal variate transformation of data led to meaningful classification of 33 different *E. breviscapus* herbal samples (see Fig. 2). The result was also colaborated by variance squares discriminant method. The quality of herbal objects was further evaluated, and the causes of this fact have been explained from a chemical point of view. The other method is based on secured principal component regression (sPCR) that was originally developed for detecting and correcting uncalibrated spectral features newly emerging in spectra after the PCR calibration. It can detect and consider unexpected chromatographic features for quality valuation of herbal samples from the point of view of analyzing fingerprint residual [Cheng, 2003; Cheng & Chen, 2003; Collantes, 1997;Vogt, 2003; Welsh, 1996; Wold, 1977; Xie, 2001].

7.7 Qualityfication and validation of two-way data from hyphenated chromatographies by chemometrics

In general, the data generated by the hyphenated instruments are matrices with every row being a spectrum and every column a chromatogram at some wavelength, wave number or m/e unit as illustrated in Fig. 6. The data obtained by such hyphenated instruments in chemistry is generally called two-way or two-dimensional data. In common, the size of the data matrix such-obtained is rather big; sometimes it can be more than 40 megabytes. Thus, this is a really a new challenge for modern analytical chemistry to deal with the chemical information embedded in it.

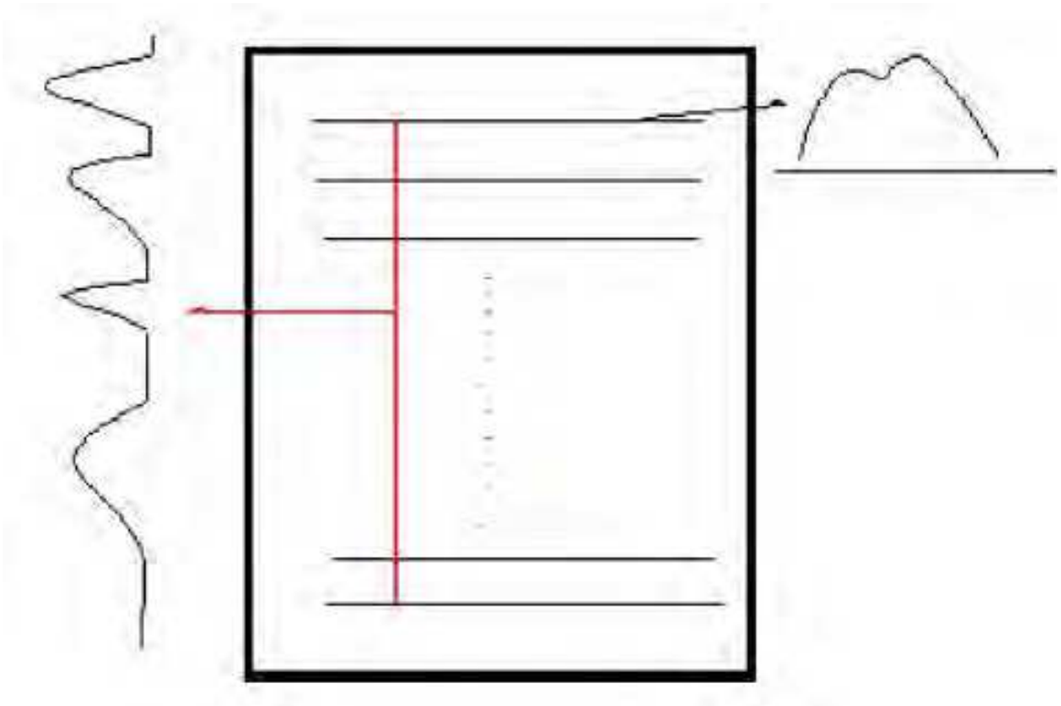


Fig. 6. Illustration of two-dimensional data from hyphenated instruments.

However, data derived from hyphenated analytical techniques have several advantages over the classic one-way chromatograms. Firstly, the two-way data matrix contains both information from spectra and chromatograms, which will make on-line structure identification of some interested compounds possible; secondly, the two-way data has so-called dimension advantages proposed by Booksh and Kowalski, which will make on-line comparison of overlapping chromatographic peaks possible; finally, the hyphenated technique might enhance the chromatographic separation ability by the additional spectral information, since one could easily find some useful component electivity with the help of chemometric local rank analysis methods, which can only be used for two-way chromatographic data but never for one-way chromatographic data. How to use these advantages from the two-way data to address the problems in evaluation of the fingerprints of herbal medicines and the problems in quality control of herbal medicines, which is just the topic we want to discuss in the following subsections. There are still many different kinds of difficult problems unsolved in the research field of herbal medicines. For instance, there may be no big problem for evaluating the fingerprints from the same instruments and/or with the same batch of the herbal products as shown above. However, if we get some fingerprints from different laboratories and/or from the same kind of column, say C-18 for example, but from different companies. Could we still evaluate them reasonably? Moreover, could we do the on-line comparison among the fingerprints of some overlapping chromatographic peaks and/or of big diversity? For instance, if we get some fingerprints from different extraction methods and/or from different herbal medicines, is it possible for us to see whether we have got the same phytochemical constituents or not in order to understand their bioactivities and possible side effects of these herbal products and consequently to enhance product quality control? With the help of the two-way data from the hyphenated chromatographies and chemometric methods recently developed, the answer is positive [Liang, 2001].

7.8 Spectral correlative chromatogram and its applications

As stated above, it is very important for assessment of the quality of various samples to determine the presence or absence of interested components among the different chromatographic fingerprints. They may be obtained either from same herbs or from different ones under the same or similar chromatographic separated conditions. Moreover, another problem is the shift of retention time of some interested peaks of various fingerprints due to inevitable possibility in quality control, such as the fingerprints from different laboratories and the experimental columns from different vendors despite the same type stationary-phase characteristics. This maybe lead to erroneous assess of quality of medical samples. Let us see an example shown in Fig. 6. $\boldsymbol{x}1$ and $\boldsymbol{x}2$ shown in Fig. 7A are the chromatograms of two dimensional data sets of $\boldsymbol{X}1$ (of the *G. biloba* samples obtained from HPLC–DAD through the column made in Angilent Inc.) and $\boldsymbol{X}2$ (through the column made in Waters Inc.) at wavelength 260 nm, respectively. It can be intuitively seen that the retention time of eluting components of them shift rather seriously, which trouble right identification of them by directly comparing their chromatographic fingerprints.

In order to deal with such a kind of problem a technique named spectral correlative chromatogram (SCC) was developed. The idea of SCC is quite simple, that is, the same chemical component should be of the same spectrum no matter what they are eluted

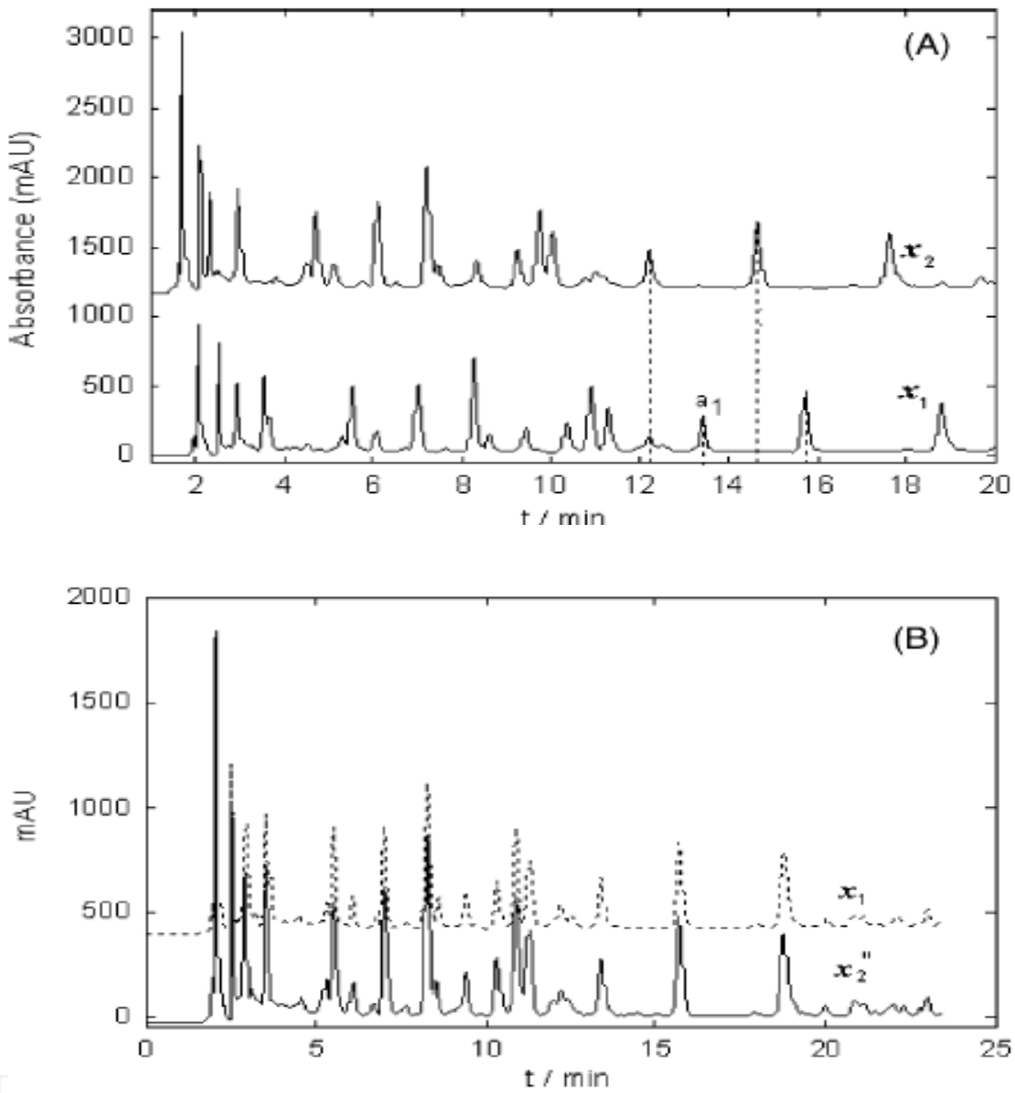


Fig. 7. Comparison of chromatograms X_1 and X_2 measure by the same kind of column but from different companies: X_1 (Angilent Inc.) and X_2 (Waters Inc.). (A) Original chromatograms of samples $X_1$and X_2 at wavelength 260nm, respectively. (B) Original chromatograms of samples X_1 and chromatogram X''_2 of sample X_2 after correction of retention time shifting by local least-square technique.

through diverse chromatographic columns. Thus, one could use the spectral information to pick up the interested compound from the other two-way chromatograms. The whole procedure goes in the following steps: (1) assess peak purity of an interested compound and then acquire its spectrum; (2) identify correlative components in the other fingerprint by series correlation coefficients between the spectrum obtained above and the spectra at every scan point for the other two-way chromatogram; (3) get a curve (named SCC) of correlation coefficients at every scan point in the direction of retention time and further validate the result from the second step combining the information of local chromatographic cluster

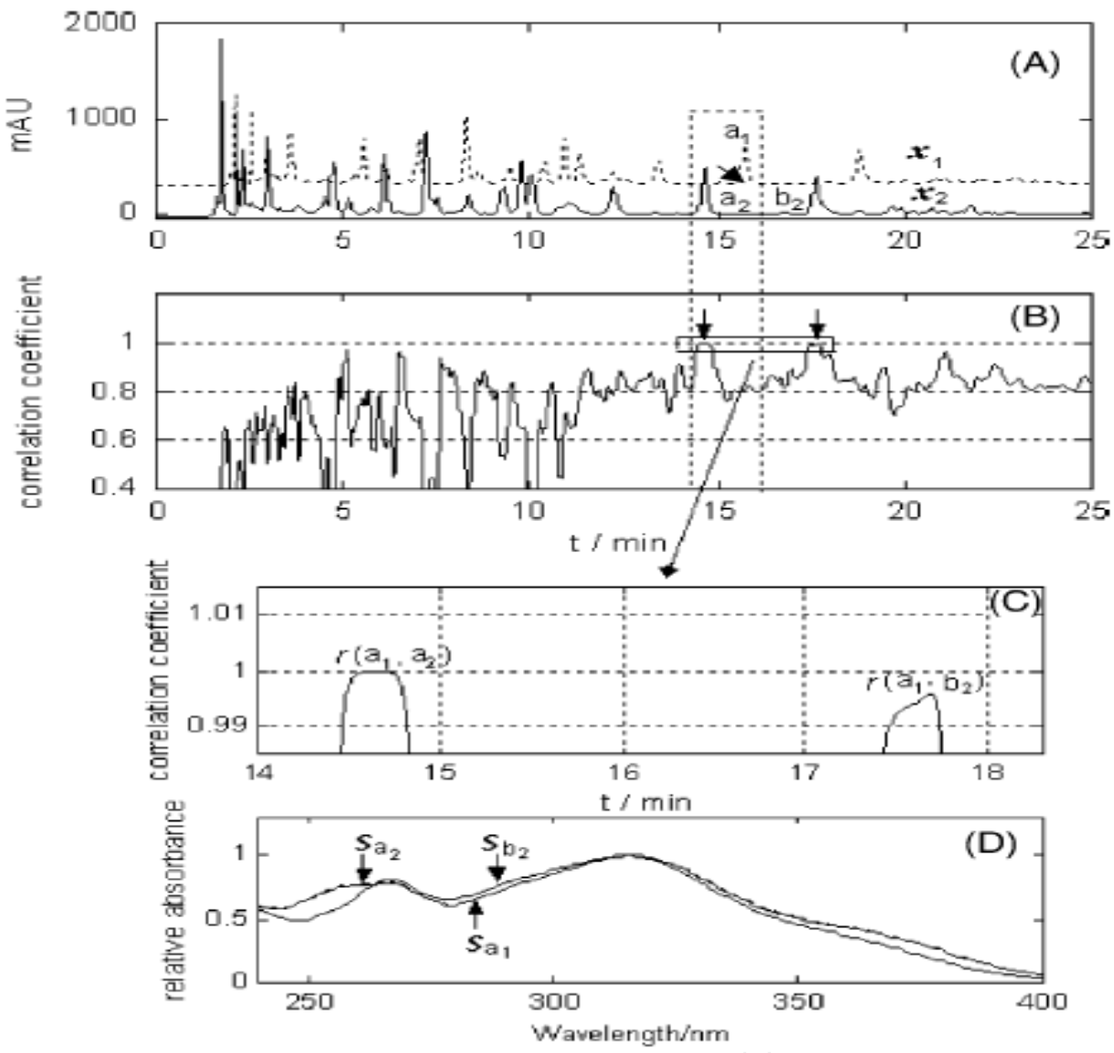


Fig. 8. Illustration of the procedure of spectral correlative chromatogram. (A) Two original samples, say $X_1$and X_2, involved in comparison, in which a peak indicated by a1 is the component spectrum to be searched by SCC. (B) The spectral correlative chromatogram obtained for component a1. (C) Enlarged part of spectral correlative chromatogram around retention time range of 14-18.5min. (D) Spectral comparision of spectra s_{a1}, s_{a2} and s_{b2} respectively.

where targets exist; (4) eventually assess the similarity and/or difference of the chromatographic fingerprints after correcting the time shift of correlative components in a piece wise way by using local least squares. Fig. 8 shows an example of this procedure. Pure component $a1$ in $\boldsymbol{X}1$ indicated in Fig. 8 was taken as an example to illustrate this procedure. The obtained SCC is shown in Fig. 8B. As could be seen from Fig. 8, both the correlation coefficients $r(a1, a2)$ and $r(a1, b2)$ corresponding to components $a2$ and $b2$ of $\boldsymbol{X}2$ were larger than others and quite close to 1, say $r(a1, a2)= 0.9998$ and $r(a1, b2)= 0.9940$. Thus, components $a1$ and $a2$ were correlative, even though they were unknown and there were some shift of their retention time. Furthermore, spectrum-dependent principle of identification of substance decided the result. Fig. 7C and D (lower part) exhibits the spectra $\boldsymbol{s}a1$, $\boldsymbol{s}a2$ and $\boldsymbol{s}b2$. It is obvious that $\boldsymbol{s}a1$ and $\boldsymbol{s}a2$ are entirely consistent and factually identical, whereas $\boldsymbol{s}a1$ and $\boldsymbol{s}b2$are something different. Similarly, other correlative components could also be obtained, as listed in Table 2. With the correlative information available, the retention time shift can be easily corrected by local least squares taking $\boldsymbol{x}1$ as a target reference. The results after shift correction are shown in Fig. 7B. From this plot, we can see that the shift of retention time can be corrected reasonably. Along with this direction, the spectral correlative chromatogram for multi-components' comparing could be also possible and conducted.

7.9 On-line comparison by chemometric methods

In order to understand the bioactivities and possible side effects of some herbal products and consequently to enhance product quality control one might be asked to compare directly some samples to see whether there are same phytochemical constituents in the different samples. For instance, if the extraction method is changed the chemical compositions of the products will also change? **Fig. 9** shows such an example, in which the total ion current chromatograms of the volatile fractions of *Schisandra chinensis* derived from six different extraction methods are presented. It may be observed that the major section (retention time region from 15 to 35 min) is quite similar in each of the six chromatograms. In order to carry out a more detailed analysis, however, it is necessary to compare qualitatively the results from the six methods.

There are three difficulties in comparing such results: (1) it is difficult to conclude if the same compounds are present in overlapping chromatographic peaks; (2) it is difficult to confirm whether a peak with a given retention time represents the same compound in different extractions or whether there is significant chromatographic drift between runs (see top part in Fig. 10 for details); and (3) the chromatographic background makes MS matching difficult. In order to overcome these problems, the mass spectral information together with chemometric methods seems to be necessary to introduced. Sub window factor analysis (SFA) is just such chemometric resolution methods, which focus its attention of comparing the spectra of two overlapping peak clusters to obtain the pure spectrum of the common component in the two overlapping peaks and further to resolve the whole overlapping peak cluster. With the help of SFA, six common components were extracted from the two peak clusters, assigned B1–B6 in the sample from extraction method 1 and b1–b6 in the sample from extraction method II (Fig. 10 (medium part) and (bottom part), respectively [Li, 2003; Liang, 1992, 1994; Karjalainen, 1992; Kvalheim ,1992; Maeder, 1987; Malinowski ,1992; Malinowski, 1991; Windig, 1997; Gemperline, 1984].

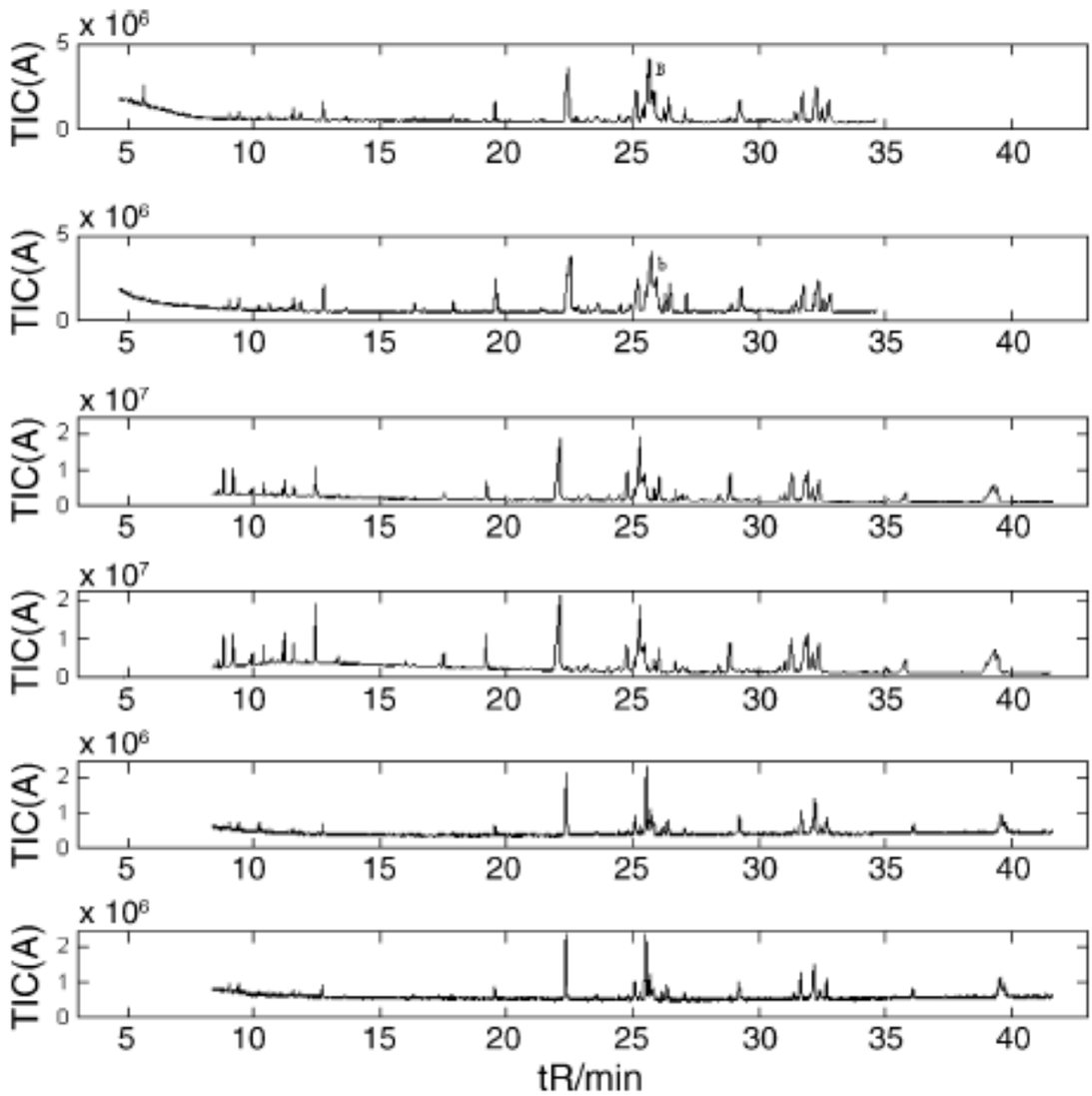


Fig. 9. Total ion current chromatograms of the volatile fractions of *Schisandra chinensis* obtained by the six different extraction methods (1-6, from top to bottom): (1) Use of the Chinese pharmacopoeia committee(1995) method for extracting essential oils from traditional Chinese medicines employing a standard essential oil extractor; (2) Steam distillation; (3) Solvent extraction with petroleum ether using an ultrasonic extractor; (4) Soxhlet extraction with petroleum ether; (5) Solvent extraction with diethyl ether using an ultrasonic extractor; and (6) Soxhlet extraction with diethyl ether.

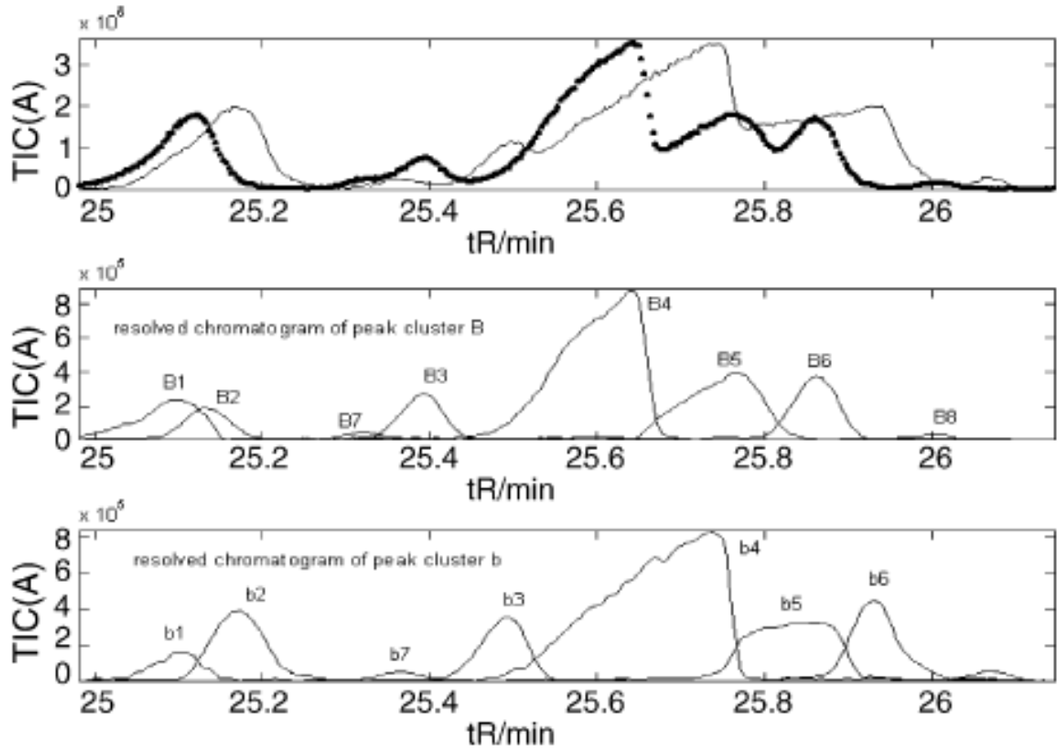


Fig. 10. Total ion current chromatograms of (top part) peak clusters B (bold dotted line) and b (thin continous line) as marked in Fig. 9 and (medium part) and (bottom part) their corresponding resolved chromatograms, respectively.

Correlation component no.	Retention time ($t1,k$) of $\boldsymbol{X}1$	Retention time ($t2,k$) of $\boldsymbol{X}2$	$\Delta t = t1,k - t2,k$ (min)
1	2.0820	1.7020	0.3800
2	2.5287	2.1153	0.4134
3	2.9953	2.4753	0.5200
4	5.5287	4.7153	0.8134
5	6.9953	6.1020	0.8933
6	8.2620	7.2087	1.0533
7	9.4220	8.3353	1.0867
8	10.3487	9.2553	1.0934
9	10.8887	9.7287	1.1600
10	11.2820	10.0287	1.2533
11	15.7020	14.6420	1.0600
12	18.7620	17.6020	1.1600

Table 2. Comparison of correlative components and their chromatographic eluting time (min) of *Ginkgo biloba* sample $X1$ with those of $X2$.

7.10 Further comments on quality control of herbal medicines

Western and traditional Chinese medical practices represent totally different philosophies. Thus, this is not a simple exercise of applying modern technologies to quality control of the products that have been in constant use for centuries. The progress on quality control of herbal medicines discussed in this review is just at its beginning stage of a long journey. Of course, the proposal of the use of chromatographic fingerprints of herbal medicines for quality control of herbal medicines is definitely a progress. However, using the chemical fingerprints for the purpose of quality control of herbal medicines can only address to the problem of comparing the integrated sameness and/or difference and controlling their stability of the available herbal products.

The complex relationship between the chromatographic fingerprints and efficacy of the herbal medicines (QRFE) is not taken into account yet, which seems to be the most important aspect for the quality control of herbal medicines. As it is well known that the efficacy of traditional herbal medicines has a characteristic of a complex mixture of chemical compounds present in the herbs, thus how to evaluate reasonably their relationship is obviously not a trivial task. THMs represent a much more daunting challenge due to the natural variability of the individual herbs and the chemical complexity of the formulations. Moreover, the chemical profile by itself is insufficient in determining the efficacy of TCM. This is where biochemistry, molecular biology, and cell biology are invaluable in establishing quantifiable and reproducible assays. Chemical fingerprints might be linked to these biological assays to provide assurance of efficacy and consistency. But the research work on this aspect, to our best knowledge, is far from sufficient to meet the criteria needed. Thus, the researches concerning the relationship between the chromatographic fingerprints and efficacy of the herbal medicines are urgent requirements for the quality control of herbal medicines. On the other hand, the works on possible contaminations in herbal products, such as excessive or banned pesticides, microbial contaminants, heavy metals, chemical toxins, should be also conducted concurrently. In fact, the research field of quality control of herbal medicines is really an interdisciplinary research. It needs crossover of chemistry, pharmacology, medicine and even statistics to provide a platform for the quality control of traditional herbal medicines and further to discover the novel therapeutics composed of multiple chemical compounds.

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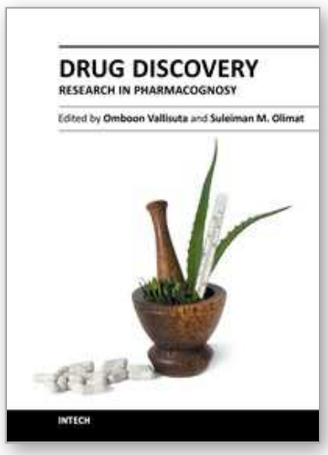
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This book, Drug Discovery Research in Pharmacognosy provides a full picture of research in the area of pharmacognosy with the goal of drug discovery from natural products based on the traditional knowledge or practices. Several plants that have been used as food show their potential as chemopreventive agents and the claims of many medicinal plants used in traditional medicine are now supported by scientific studies. Drug Discovery Research in Pharmacognosy is a promising road map which will help us find medicine for all!

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