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Advances and trends in analytical techniques in natural product research: Challenges and future perspective

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Advances in analytical methods and bioassay development have helped to push forward the research in natural products. Their high chemical diversity and the effects of evolutionary pressure to create biologically active molecules could be attributed to success in drug discovery. Despite the availability of modern analytical instrumentation techniques, metabolic profiling covers the identification of a selected group of metabolites. The phytochemical analysis is commonly performed using standard techniques such as thin-layer chromatography, high-performance thin-layer chromatography, high-performance liquid chromatography, gas chromatography and more recently mass spectrometry and nuclear magnetic resonance spectrometry. Two-dimensional *J*-resolved Nuclear Magnetic Resonance (NMR) spectra and multivariate data analysis techniques were applied to avoid low resolution and overlapping signals hampering the identification of the individual components of botanicals. On-targeted metabolomic analysis *via* the ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) was utilised to localise compounds belonging to various chemical classes (*i.e.* oxygenated fatty acids, flavonols, phenolic acids, and sinapoyl choline derivatives). Rotation planar chromatography over-pressured layer chromatography and electro planar chromatography are the other innovations. Parallel and serially coupled layers open up new avenues for the analysis of a large number of samples for high-throughput screening and very complex matrices in a natural product. Analytical strategies with applications to natural extracts and novel methods that have strong potential, regardless of how often they are used are discussed with respect to their potential applications challenges and future trends.

Keywords: Analytical techniques, Chromatography, Herbal drugs, Natural products, Spectroscopy.

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Introduction

Nature epitomizes an astonishing resource of novel molecules. Natural products have provided lead to most of the active ingredients in medicines. Their high chemical diversity and the effects of evolutionary pressure to create biologically active molecules could be attributed to success in drug discovery. Medicinal plants have played a key role in world health. Plants are rich sources of fine chemicals, largely unknown and explored, yet they still make an important contribution to health care despite great advances in the field of modern medicine. Plants are a treasure trove of interesting and valuable compounds since they must glean everything from the spot on the earth where they are rooted. Also, they cannot escape when threatened; therefore, they have evolved the most impressive panoply of products to thrive in ever-changing environments despite limitations[1]. There are about 400,000 higher plant species in the world[2]. It is estimated that plants produce up to 200,000 phytochemicals across their many and diverse members[3]. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants[4-7]. Many of these pharmaceuticals are still in use today and often no useful synthetic substitutes have been found that possess the same efficacy and pharmacological specificity to a particular disease. In some cases, such as for antitumor and antimicrobial drugs, about 60% of the currently available medicines and others in the late stages of clinical trials have been derived from natural products, mainly from higher plants[5]. Plant-based drugs and formulations have been used since ancient times as a remedy for a range of diseases. About 65–80% of the world's population from developing countries essentially depends on plants for primary health care[8]. Despite competition from other drug discovery methods, natural products (NPs) are still providing their fair share of new clinical candidates and drugs. This was demonstrated recently

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by Newman *et al*., who analyzed the number of NPs-derived drugs present in the total drug launches from 1981 to 2002[9,10]. They concluded that the NPs were still a significant source of new drugs, especially in the anticancer and antihypertensive therapeutic areas. In another study, it has been reported that 8 out of 29 small molecule drugs launched in 2000 were derived from NPs or hormones and concluded that High Throughput Screening (HTS) did not have a significant impact on the derivation of these drugs[11]. NP-derived drugs are well represented in the top 35 worldwide selling ethical drug sales of 2000, 2001, and 2002. The percentage of NP-derived drugs was 40% in 2000 and remained approximately constant at 24% in 2001 and 26% in 2002. Therefore, in addition to being a proven and important source of drug leads, NP derived drugs also contribute significantly to the profitability of many companies. Besides plants, marine natural products and microorganisms are also a major source of new drugs[12]. Oceans encompass a stressful and competitive habitat with unique conditions of pH, temperature, pressure, oxygen, light, nutrients, and salinity. These factors force organisms to adapt both chemically and physiologically to survive[13,14]. Marine natural products are potential sources for pharmaceuticals such as antimicrobial, antiviral, antiparasitic, anticancer, anti-inflammatory, neuroprotective, and immune–modulatory agents[14-19]. More than 50,000 microbial natural products also have an important role in drug discovery[20]. A renewed interest in investigating higher plants as sources for new lead structures and also for the development of standardized phytotherapeutic agents with proven efficacy, safety and quality has been demonstrated[21-23]. More than 20 new drugs that were launched globally between 2000 and 2005 originated from natural products[24].

Natural product chemistry has evolved into an interdisciplinary area of science concerned with the isolation, characterization and determination of the biological activity of pure phytochemicals, as well as extracts or enriched fractions. Phytochemicals, the active components for biological activity, are generally referred to as secondary metabolites. Plant secondary metabolites are low molecular weight compounds and can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them, but they do have an important role in the interaction of the plant with its environment[25]. They are characterized by structural diversity and are synthesized from a limited pool of biosynthetic precursors: phosphoenolpyruvate, pyruvate, acetate, amino acids, acetyl CoA and malonyl CoA. Although various hypotheses have been proposed to account for the production of secondary metabolites, none is entirely satisfactory. However, information on their biosynthesis is essential to understand the interaction between plants and the environment[26]. The production of these compounds is often low (less than 1% of dry weight) and depends on the physiological and developmental stage of the plant. Secondary metabolites are characterized by wide chemical diversity, and every plant has its own characteristic set of secondary metabolites.

Based on their biosynthetic origins, plant secondary metabolites can be divided structurally into five major groups: polyketides, isoprenoids, alkaloids, phenylpropanoids and flavonoids. It is generally accepted that plant secondary metabolites are important for the survival of the plant and in its ecosystem, their antimicrobial and anti-insect activities deter potential predators, discourage competing plant species and attract pollinators or symbionts[27]. Secondary metabolites have for centuries been of interest to humans as flavours, fragrances, dyes, pesticides and pharmaceuticals, as well as being important for plants themselves.

Herbal medicines have provided the world's populations, with safe, effective and low-cost medicines for centuries. They have a rich and extensive historical basis in use and study which can be referenced to ancient medical writings. More importantly, modern analytical research has validated many of the traditional uses ascribed to herbs. When integrated into medical care with other medications, botanicals can provide consumers and patients with the best chance for maintaining a high quality of life and, in some cases, increase their chance of survival. They can also fill therapeutic niches that are not adequately addressed through conventional therapies. As botanical supplements are integrated into the health care programs of more and more people, it becomes necessary that information regarding their optimal use be made available. Similarly, independent quality control requirements for producing herbal products need to be established to ensure that the highest degree of safety and effectiveness is achieved. Information relative to their safe clinical use, toxicology, interactions with conventional drugs, etc., is especially important to safeguard public health.

While herbal medicines are well integrated into the health care systems of many nations, authoritative information regarding the proper use and manufacture of herbal medicines is lacking. The advances in analytical methods were founded to address this deficiency.

In the field of pharmaceutical research, the analytical investigation of phytopharmaceuticals, chemical profiling of plants, degradation products, standardization and quality control and biological samples containing the drugs and their metabolites is very important. From the commencement of official pharmaceutical analysis of these natural products, analytical assay methods were included in the compendial monographs with the aim to characterize the quality of botanicals by setting limits of their active ingredient content. In recent years, the analytical methods in the monographs include titrimetry, spectrometry, chromatography, and capillary electrophoresis; also the electroanalytical methods can be seen in the literature. The present state-of-the-art analytical methods permit the incorporation of herbal monographs in various Pharmacopoeias and compendiums. From the stages of drug development to marketing and post-marketing of herbal drugs, analytical techniques play a great role, be it understanding the physical and chemical stability of the herbal drug, impact on the selection and design of the dosage form, assessing the stability of the drug molecules, quantitation of the impurities and identification of those impurities which are above the established threshold essential to evaluate the toxicity profiles of these impurities to distinguish these from that of the API, when applicable and assessing the content of drug in the marketed products. The analysis of markers and their metabolite which may be either quantitative or qualitative is extensively applied in the pharmacokinetic studies of herbal formulations. This review highlights the advances in analytical techniques and their corresponding analytical methods in natural product research and also focus on the trends challenges and future perspective of these analytical methods used in natural product research.

Natural product research and analytical techniques

Globally, there is an increasing trend towards natural product research due to a large number of opportunities, modernization of analytical tools and availability of validated methods of high-performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography mass spectrometry (GC/MS), liquid chromatography mass spectrometry (LC/MS), liquid chromatography mass spectrometry nuclear magnetic resonance (LC/MS/NMR), liquid chromatography mass spectrometry mass spectrometry (LC/MS/MS). These analytical tools are important milestones in natural product characterization, identification, and quantification. Ever-increasing costs and increasing failure at the end of discovery make medicine unaffordable to developing countries. Natural products are the new approaches that are more attractive and also with advancements in the analytical methodologies make the research more innovative for the drug discovery. It is known to all of us that the drug discovery pipeline in modern drug discovery is getting dry and this modern world is looking again towards natural products with great expectations. Traditional medicine and herbal products are generally plant-derived and consist of hundreds of unknown components rather than a single component or a simple combination of several components. Also, many of the components are low in quantity. Usually, the active principles responsible for pharmacological action are unknown. Multiple active components, including macro and micro components, are frequently considered to be responsible for the therapeutic effects, and thus the analysis of multiple components is more reasonable for quality control. Furthermore, herbal drugs, individually and in combination, contains a myriad of compounds in complex matrices in which no single active constituent is responsible for overall efficacy. Consequently, simultaneous quantitative analysis of various kinds of active components is the most direct and important method for quality control. Despite the availability of modern analytical instrumentation techniques, rarely do phytochemical investigations succeed in isolating and characterizing all secondary metabolites present in the plant extract[28]. Furthermore, only about 10% of higher plant species have been characterized chemically to some extent. Chemical complexity makes the quality control process much more complicated. However, it has become inherent to determine the chemical profile of plant-based products for better scientific and clinical acceptability, as well as for proper global positioning. Despite their existence and continued use over many

centuries and their popularity and extensive use during the past decades, traditional medicines have not been officially recognized in most countries.

The quantity and quality of safety and efficacy data on traditional medicines are far from sufficient to meet the criteria needed to support their use worldwide[29]. Furthermore, the chemical constituents in component plants may vary depending on harvest seasons, plant origin and other postharvest processes. To ensure the reliability and repeatability of pharmacological and clinical research, as well as also to understand their bioactivities and possible side effects, it is essential to determine most of the phytochemical constituents of traditional medicines[30-32]. It is also well known that the efficacy of traditional medicines has a characteristic of a complex mixture of chemical compounds present in the crude drug; reasonable evaluation of their relationship is not a trivial task. A chemical fingerprint can be linked to biological assays to assure efficacy and consistency. However, the research work on this aspect is far from sufficient to meet the criteria needed. Isolation of the ingredients of plant extracts in adequate quantities for spectral and biological assays is the basis of phytochemical research. Rapid identification and quantification of biologically active natural products play a strategic role in phytochemical investigations of crude plant extracts. Also, the dereplication of crude extracts prior to isolation work is crucial to avoid the tedious isolation of known constituents. Recent advances in the area of the purification process, isolation and structure elucidation have made it possible to establish appropriate strategies for the quality control and standardization of herbal products in order to maintain as much as possible the homogeneity of the plant extract, and therefore the derived product or formulation. A wide spectrum of analytical methods has been developed for application in phytochemical research, pharmacological studies or quality control. The analytical approaches can be classified as (i) the analysis of targeted compounds, i.e. specifically one compound or (ii) group-specific analysis, i.e. the analysis of a number of (preferably all) compounds belonging to that particular group, and metabolite profiling aimed at a large number of primary and secondary metabolites from the extract, including carbohydrates, lipids, amino acids, etc. Metabolomics involves the analysis of the entire metabolome as the sum of all detectable low and intermediate molecular mass compounds in place of individual (target) metabolites. Different approaches such as metabolite profile and metabolic fingerprinting are followed in the field of metabolomics[33]. Metabolic profiling covers the identification of a selected group of metabolites, whereas metabolic fingerprinting comprises the classification of samples based on provenance of either biological relevance or origin[34]. Metabolomics has developed into an important tool for applications in natural product studies and quality control, as well as in studies on diseases and toxicity[35]. Traditionally, histological and morphological inspections have been the usual methods of authentication. But these methods cannot be applied to the final forms of modern herbal products such as herbal extracts and dosage forms. Chemical and chromatographic techniques are currently used for the identification and assessment of components[34]. A phytochemical analysis is commonly performed using standard techniques such as thin-layer chromatography (TLC), HPLC, gas GC and, more recently, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectrometry. Significant improvements have been made in the separation and resolution of analytical techniques. High-resolution instruments are now becoming available to a broader group of researchers. Furthermore, with the developments in biostatics with the aid of multivariate analysis (pattern recognition), it is possible to compare the metabolite profile, and the differences between a group of samples can be identified rapidly, thereby rendering the identification and quantification of all individual metabolites present in the profiles superfluous.

The holistic approach requires a quite different procedure than traditional analysis, as instead of focusing on a few selected compounds, it is imperative to generate a profile of a large number of constituents, aiming at the identification of novel biomarkers and drug targets. NMR and LC-MS are the most popular techniques for metabolite profiling and fingerprinting. NMR spectra of unpurified solvent plant extracts have the potential to provide relatively unbiased fingerprints, comprising overlapping signals of the majority of the metabolites present in the solution[36]. An NMR based study on the influence of the composition of the extraction solvent in relation to the quality of the metabolite profile has also been reported[34]. Two-dimensional *J*-resolved NMR spectra and multivariate data analysis techniques were applied in order to avoid low resolution and

overlapping signals hampering the identification of the individual components of ginseng roots[37]. The elemental composition of metabolites is one of the most valuable pieces of information for identification purposes. Accurate mass measurements, which means by definition that the measured mass should be within 5 ppm of the theoretical mass, can be obtained from the latest generation Fourier transform ion cyclotron resonance (FT-ICR), thereby allowing unequivocal determination of the elemental composition. These accurate mass data are obtainable on a chromatographic timescale and without the need for internal calibration[38]. For a detailed analysis of the metabolome, chromatographic procedures are often preferred. Although there are many chromatographic techniques including hyphenated chromatography available for instrumental analysis of natural products, TLC was the common method of choice before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal products as an easier method of preliminary screening with a semi-quantitative evaluation together with other chromatographic techniques. Simplicity, versatility, high velocity, specific sensitivity and simple sample preparation are the advantages of using TLC for constructing fingerprints. Various pharmacopoeias such as the American Herbal Pharmacopoeia, Chinese Drug Monographs and Analysis, Pharmacopoeia of the People's Republic of China, etc., still permit the use of TLC for providing the first characteristic fingerprints of herbs. TLC has advantages of many fold possibilities of detection in the analysis of herbal products. Further, with the help of a video store system, it is possible to gather useful qualitative and quantitative information from the developed TLC plate[39]. TLC is also in the process of being updated. Forced flow planar chromatography (FFPC) uses hydrostatic pressure to increase the velocity of the mobile phase. Rotation planar chromatography (RPC), over pressured layer chromatography (OPLC) and electro planar chromatography (EPC) are the other innovations. Parallel and serially coupled layers open up new avenues for the analysis of a large number of samples (up to 216) for high-throughput screening and very complex matrices[40].

The methods of extraction and sample preparation are also of great importance in preparing good fingerprints. The main goal of extraction is to obtain the maximum number of metabolites or, ideally, all the metabolites present in the samples. Different kinds of extraction methods are usually used applying different solvent combinations. Soxhlet extraction is one of the oldest methods for solid-liquid extraction. It has been regarded as the reference for the optimization of new extraction techniques and has been the most cited among the other extraction techniques used. Ultrasound-assisted solvent extraction, microwave-assisted extraction and supercritical fluid extraction methods have emerged as modern extraction techniques. Solid-phase extractions, besides being used for sample cleanup or concentration of metabolites from a liquid matrix, are also utilized for extraction purposes. This technique has been automated and coupled with online extraction and analytical instruments such as GC or LC-MS[41,42]. The concept of phytoequivalence was developed in Germany to ensure the consistency of herbal products[43]. According to this concept, a chemical profile such as a chromatographic fingerprint for a herbal product should be constructed and compared with the profile of a clinically proven reference product, as it is almost impossible to develop an appropriate analytical method (including sample preparation and chromatographic procedures) to represent all the constituents of the chemical characteristics in a chromatogram[44]. Therefore, the development of multiple chromatographic fingerprints has been suggested.

Advances in hyphenation techniques in chromatographic instruments can make the quality control of natural products, both in qualitative and quantitative regards, stronger and stronger. High-throughput analysis and miniaturization that provide high resolution in a short analysis time with low operational costs are desirable. The introduction of sub-2-µm columns for HPLC and the development of ultra-high pressure liquid chromatography (UHPLC) represent important steps forward for crude extract profiling. The speed of separation provided by these new separation methods challenges the liquid chromatography detectors that have to provide faster responses. The strong development of '-omics' (metabolomics, genomics, etc.) over the last decade also challenges the analytical methods as they aim to detect and quantify all metabolites in a given organism. For these types of studies, both separative and non-separative methods must be complementary because none of them alone can fulfil all the ideal needs of sensitivity and throughput[45].

Titrimetric techniques

The origin of the titrimetric method of analysis goes back to somewhere in the middle of the 18th century. It was the year 1835 when Gay–Lussac invented the volumetric method which subsequently leads to the origin of the term titration. Although the assay method is very old yet there are signs of some modernization, i.e., spreading of non-aqueous titration method, expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric end-point detection improving the precision of the methods in natural product analysis. With the development of functional group analysis procedures, titrimetric methods have been shown to be beneficial in kinetic measurements which are in turn applied to establish reaction rates. There are many advantages associated with these methods which include saving time and labour, high precision and the fact that there is no need of using reference standards[46].

Chromatographic techniques

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 based on the partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent[47,48]. Identification can be affected 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 analyses. For each plate, more than 30 spots of samples can be studied simultaneously at one time. Thus, the use of TLC to analyze herbal medicines is still popular. HPTLC is one of the sophisticated instrumental techniques based on the full capabilities of TLC. It is the most flexible, reliable and cost-efficient separation technique. The advantage of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, and so on enable it to be a powerful analytical tool for chromatographic information of complex mixtures of pharmaceuticals, natural products, clinical samples, foodstuff, and so on. With the help of the CAMAG video store system (CAMAG, Switzerland) 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 effects compared to others as they contained the most effective component cordycepin. Moreover, with the help of image analysis and digitized techniques 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 FFPC, RPC, OPLC and 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[49-52].

High Performance Thin Layer Chromatography

With the advancement of the technique, high performance thin layer chromatography (HPTLC)

emerged as an important instrument in Plant drug analysis. HPTLC is a fast separation technique and flexible enough to analyze a wide variety of samples. This technique is advantageous in many means as it is simple to handle and requires a short analysis time to analyze the complex or the crude sample cleanup. HPTLC evaluates the entire chromatogram with a variety of parameters without time limits. Moreover, there is a simultaneous but independent development of multiple samples and standards on each plate, leading to increased reliability of results. Another advantage of HPTLC is the repeated detection (scanning) of the chromatogram with the same or different conditions. Consequently, HPTLC has been investigated for simultaneous assay of several components in a multi-component formulation[53-55]. With this technique, authentication of various species of plant extatcts as well as the evaluation of stability and consistency of their preparations from different manufactures can be done. Various workers have developed HPTLC method for phytoconstituents in crude drugs or herbal formulations such as bergenin, catechine and gallic acid in *Bergenia cilliata* and *Bergenia lingulata*[56], quercetin-3-O-β-D-rhamnoside in *Euphorbia hirta*[57], Withaferine-A in *Withania somnifera*[58], caffeine in herbal products and power drinks[59], 14-deoxy-11,12-didehydroandrographolide, andrographolide, neoandrographolide and andrographolisidein *Andrographis paninculata*[60], capsacine and piperine in Milangithailam[61], genistein and daidzein in *Glycine max*[62], bergenin in *Caesalpinia digyna*[63], artemisinin in *Artemisia annua* L.[64], betulin and betulinic acid in herbal formulation Virala[65], leuteolin in *Thymus vulgaris*[66], aescin in herbal medicinal products containing *Aesculus* and *vitis* extract[67], curcumin in herbal formulations[68], picroside-I and picroside-II in *Picrorhiza kurroa*[69], theophylline and etofylline in pharmaceutical dosage form[70].

Column chromatography

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid-liquid technique in which the stationary phase is solid and the mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent. The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of the adsorbent and the polarity of the solvent. If the activity of the adsorbent is very high and the polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of the adsorbent is low and the polarity of the solvent is high the separation is rapid but gives only a poor separation, i.e., the components separated are not 100% pure. The adsorbent is made into a slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as elution. That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called eluent. The weakly adsorbed component will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components. Intermolecular forces, which vary in strength according to their type, make organic molecules bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column[71].

Medium pressure liquid chromatography

Medium-pressure liquid chromatography (MPLC) is one of the various preparative column chromatography techniques. Separation under pressure renders the use of smaller particle size supports possible and increases the diversity of usable stationary phases. MPLC was introduced in the 1970s as an efficient technique for the preparative separation of organic compounds. MPLC overcame one major drawback of the low pressure liquid chromatography (LPLC), i.e., the limited sample loading. This separation method is now routinely used beside or in combination with the other common preparative

tools: open-column chromatography, Splash chromatography, LPLC or preparative high performance liquid chromatography (p-HPLC). The distinction between low pressure, medium pressure and high pressure LC is based on the pressure ranges applied in these techniques and the overlap is often considerable. MPLC allows purification of large compound quantities and, unlike open column chromatography and Sash chromatography, faster and improved separations are obtained. Packing of material with lower particle size under pressure enhances separation quality and the solid phase can be reused. MPLC has recently become widely used in the pharmaceutical, chemical and food industries, and many applications are found in natural product isolation. Both applications given below have been selected as examples of the transposition of analytical HPLC conditions to MPLC. The methanol extract of *Haleniacorniculata*, a Gentianaceae plant from *Mongolia,* was first passed through a Sephadex LH-20 gel column and the glycoside-rich fraction (300 mg) was then purified by MPLC on a reversed-phase RP-18 column, yielding six xanthone glycosides[72]. The search for optimal conditions was performed by analytical HPLC and was followed by direct transposition to MPLC separation[73]. The dichloromethane extract from the roots of *Tinosporacrispa* (Menispermaceae) was fractionated by centrifugal partition chromatography and one fraction was submitted to analytical HPLC with an acetonitrile gradient[74,75].

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 (3-50 μm) packing contained in a column with a small-bore (2-5 mm), one end of which is attached to a source of pressurized liquid eluent (mobile b 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 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 a better separation, some new techniques have been recently developed in the 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 (RPIPC-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 fulfil 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 the 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 herbal medicines. Moreover, the qualitative analysis or structure elucidation of the chemical components in herbal drugs 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[76-78].

Gas chromatography (GC)

GC, also known as gas-liquid chromatography (GLC), is a technique for the separation of mixtures into components by a process that depends on the distribution 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 volatile oils has numerous 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 is 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 herbal medicines. Changes in the composition of the volatile oil may also be used as indicators of oxidation, enzymatic changes, or microbial fermentation. The advantages of GC lie in its high sensitivity of detection for almost all volatile chemical compounds. This is especially true for the usual FID detection and GC–MS. Furthermore, the high selectivity of capillary columns enables the 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, liquid chromatography becomes another necessary tool for us to apply the comprehensive analysis of herbal medicines. The first fully automated online GC-IR system was developed by Scott *et al.*[61] Each eluted solute was adsorbed in a cooled packed tube, and then thermally regenerated into an infrared vapour cell. Subsequent to the IR spectrum is obtained, a small sample of the vapour was drawn from the IR cell into a low-resolution mass spectrometer and the mass spectrum was also taken.

Liquid chromatography-capillary electrophoresis

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. Artifacts in CE measurements could be overcome with the help of some information handling techniques, such as some chemometrics methods because they could be combined with additional information from spectra to correct for artefacts caused by the chosen separation buffer chemistry or hidden instrumental constraints. In sum, as the hyphenated techniques in chromatographic and electrophoretic instruments develop our ability to analyse herbal medicines, both in qualitative and quantitative respects and our ability of quality control of herbal medicines will become stronger. We are quite sure that we will have a very prospective future for quality control of herbal medicines 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 the production of ions depending on their mass and charge. It is widely used in quantitative determination and analysis particularly assay development and trace level determination. When MS is linked to CE then it produces determination of the molecular weight of components often termed as CE-MS. Separation is achieved from the etched surface of the capillaries that delivers the sample to the ESI MS. This technique runs in full automation and has a higher sensitivity and selectivity. The new interface known as coaxial sheath interface was developed which has the potential use in both CE-MS and LC-MS alternatively on the same mass spectrometer.

Ultra performance liquid chromatography

In 2004, separation science was revolutionized with the introduction of Ultra-Performance Liquid Chromatography (UPLC). Significant advances in instrumentation and column technology were made to achieve dramatic increases in resolution, speed, and sensitivity in liquid chromatography. For the first time, a holistic approach involving simultaneous innovations in particle technology and instrument design was endeavoured to meet and overcome the challenges of the analytical laboratory. This was done

to make analytical scientists more successful and businesses more profitable and productive.

The UPLC system allows shortening of analysis time up to nine times compared to the conventional system using 5 μm particle packed analytical columns. In comparison with 3 μm particle packed analytical columns analysis could be shortened about three times. The negative effect of particle decrease is back-pressure increase about nine times (versus 5 μm) or three times (versus 3 μm), respectively. The separation on UPLC is performed under very high pressures (up to 100 MPa is possible in the UPLC system), but it has no negative influence on the analytical column or other components of the chromatographic system. Separation efficiency remains maintained or is even improved.

Ultra-high performance liquid chromatography

Reducing the particle diameter from 5.0 μm to 1.7 μm will, in principle, result in a 3-fold increase in efficiency, 1.7-fold increase in resolution, 1.7-fold in sensitivity, and 3-fold increase in speed. For fast analyses using sub-2 μm particle column dimensions are typically 50x2 mm. An additional benefit of UHPLC is the low consumption of the mobile phase, where it saves at least 80% compared to HPLC. The high back-pressure resulting in decreased particle size need an appropriately designed chromatographic system that would withstand such high pressure (instruments nowadays up to 1200 bars) and also provide at least possible extra column effects. To prevent clogging, manufacturers of UHPLC recommend filtration of both samples and solvents through a 0.2 μm filter. Advantages as enhanced separation efficiency, short analysis time and high detection sensitivity make UHPLC coupled with MS/MS an even more powerful analytical support in pharmacokinetic studies.

Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry

This application describes a novel approach to plant drug metabolism using UPLC coupled to a hybrid quadrupole orthogonal time of flight (Q-TOF) mass spectrometer. As technology progresses, better levels of detection are obtainable not only via a more sensitive assay but through better quality data. Using UPLC with QTOF-MS adds a new dimension to metabolism studies of natural products, enabling attainment of better detection limits, better throughput, and increased chromatographic resolution, which in turn will improve data quality from the mass spectrometer. An ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS)-based metabolomics approach was developed to evaluate the holistic qualities and to explore characteristic chemical components of commercial White Ginseng and Red Ginseng. Through unsupervised principal component analysis (PCA) and supervised orthogonal partial least squared discrimination analysis (OPLS-DA) of the data from UPLC-QTOF-MS/MS, holistic quality inconsistencies of commercial WG and RG were identified, and the possible reasons involved were deduced by further elucidating the characteristic components of the groups. *Glechoma longituba* is a widely used traditional Chinese medicine (TCM) in treating various diseases; however, the *in vivo* integrated metabolism of its multiple bioactive components remains unknown. In this paper, ultra-performance liquid chromatography (UPLC) coupled to a quadrupole time-of-flight (QTOF) and the MetaboLynx™ software combined with mass defect filtering (MDF) together provide unique high throughput capabilities for drug metabolism study, with excellent MS mass accuracy and enhanced MS^E data acquisition. This rapid automated analysis method was successfully applied for screening and identification of the constituents absorbed and metabolized studies of *G. longituba* extract after oral administration to rats. *Nigella sativa*, commonly known as black cumin seed, is a popular herbal supplement. Large scale metabolic profiling including UPLC-PDA-MS and GC–MS with further multivariate analysis was utilized to classify 6 *Nigella* species. Under optimized conditions, the authors were able to annotate 52 metabolites including 8 saponins, 10 flavonoids, 6 phenolics, 10 alkaloids, and 18 fatty acids.

Monolithic chromatography

The use of a single rod monolith column is an alternative approach to the chromatographic columns packed with fine particles. The high permeability allows the use of higher flow rates and therefore shorter chromatographic runs. High flow rates may require flow splitting before entering MS. An attractive approach using monolith separation is to combine it with high flow online extraction, which allows fast extraction and separation of samples[77]. Current limitations in the application of these columns are the small pH range[2-8], poor temperature resistance,

limited column dimensions and stationary phases (C8 and C18) as well as higher costs due to higher mobile phase consumption.

Rotation planar chromatography

The versatile novel instrument for rotation planar extraction and rotation planar chromatography was exploited for the investigation of oak bark (Quercus robur L.). The same instrument enabled the extraction of the bark, analytical proof of (+)-catechin directly in the crude extract and also its fractionation. Additionally, epimeric flavan-3-ols, (+)-catechin and (-)-epicatechin were separated by analytical ultra-micro rotation planar chromatography on cellulose plates with pure water as developing solvent.

Over pressure layer chromatography

Over Pressure Layer Chromatography (OPLC) is a powerful separation technique that employs a planner sorbent in a pressurized chamber. The flat column is pressurized to 5 MPa (50 bars) and the mobile phase is forced through it at constant linear velocity via a pump. Bonded phase and normal phase silica with particles size as low as 5 μm are available. OPLC provides superb separations thanks to its high resolution of up to 17000 theoretical plates. The aspects that make OPLC a preferred technique for many of these assays include limited sample preparation, the semi-disposable nature of the column, high capacity suitable for semi-quantitative analysis and micro-preparative scaleup multiple parallel samples in a single run, and the possibility of direct on-column detection. OPLC technology has integrated several disciplines including pharmacognosy (plant research). However, many specific applications have been explored over the past 20 years. These include the detection of drugs and metabolites in animal tissues (homogenates, urine), potentially active ingredients in plant extracts etc.

The separation of a broad range of plant extracts including coumarins, flavanoids, anthocyanins, and cannabinoids have been performed by OPLC. These separations use polar stationary phases and moderately polar mobile phases to provide rapid, well-defined separations on the analytical as well as semi-preparative scale, so that scale up and isolation of the active components is readily affected. detection of the various compounds is directly performed on the OPLC sorbent bed via a densitometer, further simplifying the overall analysis.

Spectroscopic techniques

Ultraviolet- visible spectroscopy

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared [NIR]) ranges. The absorption or reflectance in the visible range directly affects the perceived colour of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state. Many natural phenolic compounds have been investigated by UV-Vis spectroscopy techniques.

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a measurement technique that allows one to record infrared spectra. Infrared light is guided through an interferometer and then through the sample (or vice versa). A moving mirror inside the apparatus alters the distribution of infrared light that passes through the interferometer. The signal directly recorded, called an "interferogram", represents light output as a function of mirror position. A data-processing technique called Fourier transform turns this raw data into the desired result (the sample's spectrum): Light output as a function of infrared wavelength (or equivalently, wave number). As described above, the sample's spectrum is always compared to a reference.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy, most commonly known as NMR spectroscopy, is a research technique that exploits the magnetic properties of certain atomic nuclei. This type of spectroscopy determines the physical and chemical properties of atoms or the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its functional groups.

Most frequently, NMR spectroscopy is used by chemists and biochemists to investigate the properties of organic molecules, although it is applicable to any kind of sample that contains nuclei possessing spin. Suitable samples range from small compounds analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy to large proteins or nucleic acids using 3 or 4-dimensional techniques. The impact of NMR spectroscopy on the sciences has been substantial because of the range of information and the diversity of samples, including solutions and solids.

Mass spectroscopy

Mass spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions. The information contained in a single mass spectrum, recorded using very small amounts of sample, will often allow the unambiguous identification or structural elucidation of an unknown compound. The development of ionisation techniques that do not require the sample to be liquefied before ionisation has made mass spectrometric analysis possible for a wide range of biomedical and biological molecules. The high sensitivity and specificity of the mass spectrometer are enhanced by using a gas chromatograph as the mass spectrometer inlet, and combined gas chromatography-mass spectrometry (GCMS) is one of the most powerful analytical techniques available today. The combination of the very high sensitivity and specificity of GCMS is unobtainable even with highly sensitive immunological techniques. A number of major developments have occurred in recent years in GCMS methodology. These include the direct coupling of capillary gas chromatographic columns to mass spectrometers, the widespread availability of ion sources for chemical ionization, and the increasing use of isotopically labelled internal standards in assays using quantitative selected ion monitoring (QSIM). A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. The spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds. Mass spectrometry works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios.

Liquid chromatography- mass spectrometry instrumentation

Ionization techniques

A LC-MS ion source has the double role of eliminating the solvent from the LC eluent and producing gas-phase ions from the analyte. The application of atmospheric pressure ionization (API) methods has provided a breakthrough for the LC-MS systems and has brought them to the forefront of analytical techniques in natural product research. Some ion sources such as API operate at atmospheric pressure whereas others like electron impact (EI) or chemical ionization (CI) operate in a vacuum. While soft API interfaces, in particular electrospray, produce molecular ions with minimal fragmentation, high energy sources like EI mostly generate fragmentations. API techniques are most widely used for metabolite detection, identification and quantification due to the ability to operate at atmospheric pressure, good compatibility with reversed phase chromatography and generation of intact molecule ions at very high sensitivity. All three API techniques: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are complementary.

Electrospray ionization is by far the preferred method for metabolite identification and quantification. It is the most universal technique for introducing the molecules into the gas phase and it is most gentle and therefore likely to yield intact molecular ions. ESI is ideally suited for polar, ionic and thermally labile compounds such as drug metabolites; in particular glucuronides and others phase II metabolites. This technique requires ionization of analytes within solution prior to introduction into ion source and thus works best for fairy basic or acidic compounds. Depending on the voltage polarity, nebulised droplets trapping the ionized analyte will be positively or negatively charged. The reduction in size caused by solvent evaporation accounts for the increase in charge density in the droplet leading to its explosion when repulsive forces between charges exceed the cohesive forces of the droplet. This process occurs repeatedly until gas phase ions are produced. Ions in solution are emitted into gas phase without application of heat making ESI suitable for analysis of thermo labile compounds. Many parameters, such as analyte and solution characteristics: pKa, analyte concentration, other electrolytes in solution, dielectric constant of the solvent, affect the ion formation process.

Atmospheric pressure chemical ionization is more suited for less polar compounds. Certain classes of the compound such as heavily halogenated chemical compounds and highly aromatic compounds will run readily on APCI while giving no or a weak response on ESI. APCI like ESI produces ions based on the API strategy but through a completely different process. Here, the liquid eluent is sprayed into heated chamber (450-550 °C) where the high temperature of a nebulizer gas flow causes the immediate evaporation of the solvent and the analyte. In addition to volatility at the applied temperature, thermal stability of the analyte is also a prerequisite for the successful application of APCI (e.g. glucuronides may break down and appear in the form of protonated aglycone. Ionization of analytes takes place in gas phase where due to the high flux of electrons from corona discharge needle, solvent molecules initially react with electrons and form ions that produce protonated solvent ions through secondary reactions. These protonated solvent ions then transfer a proton to form protonated analytes. For efficient ionization, the employed mobile phase should be volatile and also amenable to gas phase acid-base reactions. APCI technique is less prone to ion suppression and provides a wider dynamic detection range than ESI due to ionization that occurs mainly in gas phase. Also, a typically higher flow rate is used with APCI [1-2 mL/min) than that in conventional ESI (0.1-0.5 mL/min).

Atmospheric pressure photoionization is a relatively new ionization method. This technique can be used for ionization of analytes that are not easily ionizable by ESI and APCI. APPI has a similar application range as APCI but slightly extended toward nonpolar compounds. The APPI ion source is very similar to APCI source, except the APCI corona discharge needle is replaced by photoionzation lamp. Depending on the analyte proton affinity relative to the composition of the mobile phase, either a radical molecular ion (typically for nonpolar compounds) or a protonated molecular ion (typically for polar compound) is obtained. APPI has a potential in the analysis of drug metabolites but more research is needed to fully understand the important parameters and factors that affect ionization efficiency.

Mass analyzers

The function of mass analyzer is the separation of ions formed in ionization source according to their different mass-to-charge (m/z) ratios. The quality of mass separation is characterized by the degree to which close m/z values can be separated in the mass analyzer. Mass analyzers are classified regarding resolution into low and high resolution instruments. The later ones are associated with another important parameter, mass accuracy, which allows determination of elemental formula of particular analyte. The selection of suitable analyzer is driven by the purpose of the analysis and the instrument performance but also depends on the instrument availability and cost effectiveness.

Triple quadrupole instruments (QQQ) are the most common mass spectrometers in analytical laboratories, having most often been acquired for their evident strengths in high sensitivity quantitative analysis of known analytes. These instruments have been often applied also for metabolite identification due to wide availability and excellent tandem mass (MS/MS) properties. In QQQ, the first quadrupole filters ions of interest, the second quadrupole also called collision cell fragments these ions and further the fragment ions are filtered by third quadrupole before reaching the mass detector. Such QQQ configuration allows performing different scans such as full scan, product ions can, precursor ion scan (PI), constant neutral loss scan (CNL), single ion monitoring (SIM) and selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). PI and CNL are particularly useful in metabolite identification since both scanning modes do not require previous knowledge about the molecular weight of metabolites. High sensitivity for quantitative purposes is retained only when working in MRM mode, however, the detection sensitivity decreases dramatically when wide mass range is analyzed in a scanning mode. This is one of the major disadvantages of using QQQ for the screening of drug metabolites.

Ion trap instruments (IT) are like QQQ relatively inexpensive and compatible with wide range of ionization interfaces. These analyzers utilize ion trap chamber where ions are trapped and then selectively ejected from the chamber. Additionally, the resonance excitation applied in the trap provides efficient dissociation of the precursor ions to product ions. IT provides more sensitivity for structural elucidation than QQQ due to its better sensitivity in full scan mode and efficient dissociation of the precursor ions which allows multiple stages mass spectrometry (MSn). Recently, to address classical ion traps (called also 3D IT) shortcomings of insufficient ion storage

efficiency, capacity and deterioration of the mass spectrum and dynamic response range, linear IT has been developed. The detection sensitivity in linear IT is at least two orders of magnitude higher than that in 3D IT. Because of these advantages, linear IT will probably in near future totally replace old 3D IT.

Triple quadrupole-linear ion traps (QTrap) combine sensitive QQQ technology with high capacity of linear IT incorporating high trapping efficiencies. In this instrument, the last quadrupole of QQQ is replaced with a linear ion trap, which operates as a mass resolving quadrupole or a linear ion trap. This provides clearly increased metabolite screening capabilities compared to traditional IT or QQQ. QTrap enables high sensitivity, wide range mass scanning and MSn together with QQQ capabilities, such as PI, CNL and very high sensitive MRM data acquisition.

Time of flight (TOF) analyzers are the most suitable high resolution mass spectrometers for fast and cost-efficient metabolite identification. TOF are relatively simple and capable of recording all formed ions on a microsecond time scale offering high sensitivity detection. Ions are accelerated from the ion interface to a fixed kinetic energy and then pass through a field-free tube to the detector. The time needed for ion to reach the detector is proportional to its m/z ratio. TOF strength lies in its very high detection sensitivity when acquiring wide range data, enabling the simultaneous detection of data for all metabolites of interest in one run. High mass resolution and mass accuracy (< 3-5 ppm) enable reliable and accurate identification of metabolites by determination of elemental formula of a metabolite. Additionally, the very high acquisition speed makes them ideal for fast chromatography.

Triple quadrupole-time of flight (Q-TOF) instruments combine first mass filter and collision cell of QQQ with TOF as the second mass analyzer. These instruments can operate as true tandem MS while providing accurate mass of the product ions. Most modern Q-TOFs have good linear response and are therefore also suitable for quantitative purposes. However, TOF instruments have not the ability to perform positive/negative switching in one run.

Orbitrap is another high resolution analyzer which is a hybrid composed of a linear IT and Fourier transform mass spectrometer. It is an effective alternative to the TOF instruments used for metabolite profiling. Orbitrap is capable of high sensitivity screening over wide mass range, MSn and tandem mass spectrometry with accurate mass data for both parent and fragment ion. However, it is not suitable for fast chromatography because it suffers from a slow data acquisition.

Fourier transform-ion cyclotron resonance (FT-ICR) is the third high resolution mass analyzer. The high sensitivity, accurate mass measurements, high mass resolution and MS/MS capabilities of FT-ICR make it attractive for structural determination of ions. However, the combined requirement of ultra-high vacuum system, super conducting magnets as well as sophisticated data system place the cost of these instruments beyond the means of most laboratories involved in drug metabolism studies.

Gas Chromatography-Mass Spectrometry

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

Liquid Chromatography and Nuclear Magnetic Resonance

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 the 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.

Challenges to natural products-based analysis

In spite of the success of the traditional approach to drug discovery by the bioactivity directed fractionation of plant and marine extracts, this approach has not fared well in recent years, particularly in terms of funding from the major granting agencies in the U.S. and Europe, and in the support of this research within major pharmaceutical companies. The major reasons for this can be summarized as follows:

Incompatibility of crude natural product extracts with high-throughput screening

Drug discovery within the pharmaceutical industry, with few exceptions, is based on the high through put screening (HTS) of tens of thousands of compounds a week, using enzyme or receptor-based assays designed to uncover compounds with specific mechanisms of action[50]. This poses a dual problem for natural products screening. In the first place, crude natural product extracts are complex mixtures, containing hundreds of compounds, often including polyphenolic compounds such as plant tannins. Tannins act as promiscuous protein binders, and thus give false positive readouts in HTS, so that crude plant extracts cannot be used in HTS. Although this problem is solvable in principle by detannization procedures[51], a second problem then rears its head. Once a lead extract has been identified in natural products drug discovery, in the classical approach the active compound must be isolated by a process of bioactivity-directed fractionation, which can take weeks or months. HTS is not a good mechanism to use for this approach, because a typical HTS assay may be online for only a few weeks, and so the fractionation would need to be supported by another assay, adding cost to the process.

Diversion of resources to combinatorial chemistry

The increasing availability and sophistication of HTS from the early 1990's created the opportunity to screen libraries of hundreds of thousands or even millions of compounds, far larger than the existing compound libraries at most major pharmaceutical companies. This naturally created a demand for compounds to satiate the maw of the screening monster, and combinatorial chemistry provided the perfect fit, with its ability to generate libraries of tens of thousands of compounds. It was seemingly a marriage made in heaven. Sadly, this approach has not been the panacea that it was hoped to be, and few drugs have been discovered by the combination of HTS and combinatorial chemistry. This lack of productivity is in part responsible for the decline in new drugs, with only 20 new drugs approved in the USA in 2007, down from an average of about 40 a year from 1981–2005[38] . Although the productivity of combinatorial chemistry as a drug discovery tool will no doubt eventually improve, as more importance is being placed on making "natural product like" compounds by diversity-oriented synthesis the present situation has not changed significantly since 2004, when Ortholand and Ganesan could write: "The early years of combinatorial chemistry suffered from an excess of hype, and a major victim was natural product screening. Many organizations went through an irreversible shift in policy, and prematurely discontinued their efforts in this area. We are now seeing the backlash from thisknee-jerk reaction. The early combinatorial strategies were flawed and unproven, and have yet to deliver any blockbuster drugs. Meanwhile, we have lost the uniqueness of screening natural-product space as a complement to synthetic compounds. If past indicators are any guide, there are undoubtedly many more unique and potent biologically active natural products waiting to be discovered. A recent review by Ganesan concludes

"one can only hope that natural products that have served as an important source of drugs in the past will not be overlooked in 21st century drug discovery.

Technical difficulties

In addition to the problems with HTS noted above, the isolation of bioactive compounds from plants and marine organisms faces a number of technical challenges. These include the variability of the source material (since an activity found in one collection may be absent in another), the difficulty of isolating the active constituents, the possibility that the active compound is a known compound (thus not protectable by composition-of-matter patents), and the costs of collection. However, as will be discussed below, new methods and techniques offer exciting opportunities to avoid or at least ameliorate many of these difficulties.

Resupply problems

A further level of difficulty is encountered once a particular natural product has been isolated and identified as a lead compound since this raises the large issue of compound supply. Depending on the potency of the compound and its target, several grams to hundreds of grams are needed for preclinical development, and multi-kilogram quantities would be needed for clinical use. Probably the classic case of the problem of compound supply was with the anticancer drug paclitaxel, then known as taxol. The clinical activity of this compound against ovarian cancer was reported in 1989 and this touched off an intensive search for supplies for clinical use in what has been called the "taxol supply crisis". The problem was especially acute in the case of taxol because it treated a life-threatening disease but was obtainable at that time only from the bark of the western yew, *Taxus brevifolia*, which grew predominantly in the old-growth forests of the Pacific Northwest, home to the endangered spotted owl. The solution to this problem initially involved synthetic chemistry, as described below.

A different kind of resupply problem arises when the plant itself is used as the medicinal agent, as is still the case for a large percentage of the world's population. In this case, there is a real danger that non-sustainable harvesting will result in depletion of these critical resources, and initiatives are needed to commercialize the cultivation of the major species involved. This aspect of the supply problem is discussed in more detail by Cordell.

Policy issues

The access and benefit sharing (ABS) provisions of the Convention on Biological Diversity (CBD) could be construed as an impediment to making natural product collections outside the researcher's home country, and it cannot be denied that the legal requirements involved in meeting its terms can be time-consuming. There is also concern that these provisions will limit academic researchers interested in non-commercial studies such as taxonomy, ecology, and evolutionary biology. However, these provisions should be viewed as an opportunity to carry out natural products research in an ethical way, within an agreed legal framework. In this sense, it protects the institution or company involved from charges of biopiracy, and in addition, provides the possibility of doing some real good for a developing country. These issues will be discussed in more detail below.

Financial pressures

On top of all the problems noted above, the pharmaceutical industry in general, and particularly in the USA, is undergoing a massive retrenchment, with major cuts in pharmaceutical research and development. As one analysis put it, "Big pharma's path through the recession is littered with job and program cuts and plant closures, and lists numerous examples to back up this statement. These financial pressures make it very difficult for "Big Pharma" to invest the resources that would be needed to regain the effectiveness of their former natural product discovery programs. This in turn implies that developing nations cannot rely on "Big Pharma" to discover and develop their medicinal natural product resources; this task must be undertaken by smaller and more nimble companies and by academic researchers.

Forms of natural products and good manufacturing practices

There are several common forms of natural products, including phytochemicals, nutraceuticals, cosmeceuticals, oleoresins, essential oils, etc. Phytochemicals refers to the chemicals present in plants. Nutraceuticals are any substance that may be considered as a food or a part of food providing health benefits, including the prevention and treatment of diseases. Oleoresins are pure extractives derived from spices containing concentrated natural flavouring components both volatile and non-volatile.

Cosmeceuticals is a term to describe cosmetic-containing ingredients. The volatile part of the plant largely responsible for its characteristic aroma comes under the category of essential oil. Good manufacturing practice (GMP) is a code of practice used for maintaining the highest standard of quality in the process of the production and control of natural products, in particular in herbal products.

Future perspective

Drug discovery and development is an extremely complex, technology and capital-intensive process that is facing major challenges with the current target rich–lead poor situation. A major cause of attrition in drug discovery is due to toxicity in human trials and it is known that drugs with novel mechanisms have higher attrition rates. Better validated preclinical targets with proof-of-concept of better efficacy and safety of drugs can, however, mitigate such attrition risks. We propose that the reverse pharmacology approach can be useful in this process and help in reducing failure rates.

Seeking new synergistic combinations and improvements in bioavailability are innovative strategies that can play a significant role in drug development. For instance, in animal studies, a combination of artemisinin derivative and curcumin has been reported to show a synergistic interaction in killing *Plasmodium falciparum* leading effectively to total survival. There have been several studies on piperine showing its combination improved bioavailability of synthetic drugs such as propranolol, theophylline and rifampicin. The clue for piperine as a bio enhancer came from Ayurveda. Such bioavailability enhancing activity may have numerous advantages in drug development including reduction in dose, toxicity and treatment costs. Herbalome is an ambitious project from China that is expected to undertake high-throughput screening, toxicity testing and clinical trials to identify active compounds and toxic contaminants in popular recipes to identify scores of drug candidates. It is believed that drugs based on traditional medicine may provide a cost-effective alternative to protein-based or other biotech-based expensive therapeutics. Multisite mechanisms of action of herbal preparations from the crude extracts may offer greater chances for success where conventional single-site agents have been disappointing. Single drugs, however, may not be an optimal way to treat patients, with so many characteristics that are so individual, associated, of course with the challenges of genetic diversity. Genome-wide functional screening against disease targets may be the practical approach. Combining Ayurveda and functional genomics in a systems biology scenario may reveal the pathway analysis of crude and active components. Pharmacogenomics is now significantly influencing drug discovery and genotyping is recommended for drugs that are metabolized by enzymes whose genes have inactivating polymorphisms. Efforts to correlate genotype and phenotype-based traditional methodology of classifying humans into three major Priority types or constitutions described in Ayurveda have opened an exciting scientific chapter and will help the progress of individualized medicine approaches. The issue of protecting intellectual property rights poses special challenges in such approaches based on natural resources. The Traditional Knowledge Digital Library (TKDL) developed by CSIRand AYUSH offer unique technologies.

The TKDL has been able to provide a scientific classification structure to traditional knowledge, resulting in an altogether new resource classification system. The issues relating to patenting of new products that rely on old knowledge are also important. The European Patent Office has been recently given access to the TKDL database so that patent applications relying on Indian traditional remedies can be blocked at an early stage. Similar arrangements with the US Patent Office and others are also underway. Another ambitious project named AyuSoft involves systems standardization for an integrated, intelligent and communicative decision support system (http://ayusoft.cdac.in/). AyuSoft has converted classical Ayurvedic textual knowledge into comprehensive, authentic, intelligent and interactive repositories with complex analytical tools that can be used as a powerful discovery resource. A systematic medicinal plants database also remains very crucial and important tool for bioprospecting. NAPRALERT –a relational database of natural products developed by the University of Illinois at Chicago (http://www.napralert.org/) and the online Encyclopedia of Indian Medicinal Plants developed by the Foundation for Revitalization of Local Health Traditions (FRLHT) now known as Indian Institute of Ayurveda and Integrative Medicine (IIAIM), Bangalore, India (http://www.frlht.org.in/) are two

most available valuable resources. The World Health Organization's Commission on Intellectual Property and Innovation in Public Health has also recognized the promise and role of traditional medicine in drug development for affordable health solutions. Many countries for example India, China, Korea, Malaysia, Brazil, South Africa, Australia and the like are becoming increasingly aware of the value of their traditional knowledge. On the other hand, the global pharmaceutical industry is looking for innovative solutions to expedite the discovery process. Therefore, innovative approaches inspired by traditional knowledge like Ayurveda may aptly occupy this niche strategy to expedite the drug discovery and development process, especially in the existing global economic environment. Admittedly, despite the vast potential and possibilities, as of now, very few success stories have emerged from Ayurveda. This may be because most of the work in this field has remained within the clinics of traditional practitioners or confined to academic research laboratories and not taken seriously by industries that are strong in research and development. Therefore, path-breaking initiatives like NMITLI in India are crucially important. The Government of India's golden triangle project integrating biomedicine, modern sciences and traditional medicine is indicative of attract where traditional sciences like Ayurveda are increasingly embracing the scientific evidence-based and the spirit of robust search. Finally, after this discussion of traditional medicine-inspired approaches to drug discovery, we return to the question posed in the title: Can Ayurveda show the way forward? We can offer an assertive answer, albeit with caveats. Great traditions like Ayurveda and TCM certainly offer sound rationale, valuable experiential wisdom and a large database of botanical resources. For several reasons, researchers involved in modern drug discovery have started revisiting ancient traditional knowledge and ethnopharmacology, especially to develop new, effective synergistic drug combinations for the management of difficult to treat conditions like cancer and dementia. Many promising leads like curcumins, withanolides and the like offer great hope, and need to be taken to their logical conclusions. A paradigm shift in innovation strategy is needed, involving, among other things, revisiting the vast possibilities that the Ayurveda and other global traditional knowledge bases offer. A strong conduit between Ayurveda, academia and industry coupled with robust evidence-based research and mutually respected public-private partnership is possibly the right way forward.

Conclusion

Analyzing chemical markers that are known to be present in the natural product is one of the common quality control methods used in research laboratories in the industry. Variances duet geographical source, cultivation and processing methods affect the chemical composition and clinical efficacy. Therefore, it is necessary to establish a method to control the quality and purity of natural products. Furthermore, multi-sourcing has been a major cause of clinical accidents in physiotherapies. Physically similar plants from the same or even different genera are used as the same herb. The differences in the chemical compositions of various species may lead to different biological activities. HPLC is efficient in separating the chemical compounds in a mixture and, using mass spectrometry, sufficient information for structural elucidation of the compounds could be generated when tandem mass spectrometry (MSn) is applied. Therefore, the combination of HPLC and MS facilitates the rapid and accurate identification of chemical compounds, especially when a pure standard is unavailable. The identification of the compound is confirmed using UV, FTIR, NMR and MS.

Conflict of interest

The author declare that there is no conflict of interest.

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