Biological Fingerprinting Analysis of Traditional Chinese Medicines with Targeting ADME/Tox Property for Screening of Bioactive Compounds by Chromatographic and MS Methods

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Abstract: Traditional Chinese medicines (TCMs) are attracting increased global attention because of their potential to provide novel therapeutic agents based on substantial historical records of efficacy in man. Many strategies have been designed for the screening and selection of bioactive compounds from these complex natural products mixtures. Biological fingerprinting analysis (BFA), based on small molecule-biomacromolecule interactions in complex systems, has been applied to screen the multiple bioactive compounds in natural products. Here we review the chromatographic and MS approaches used for BFA of natural products with targeting absorption, distribution, metabolism, elimination and toxicity (ADME/Tox) properties. Such chromatographic methods cover a wide range of applications including liposome, serum proteins, liver homogenate and DNA profiling. MS methods for the characterization of molecular interactions between natural products and target molecules by ESI and MALDI-TOF MS are also discussed.

Key Words: Biological fingerprinting analysis (BFA), natural products, traditional Chinese medicine, screening, bioactive compounds, ADME/Tox, small molecule-biomacromolecule binding.

1. INTRODUCTION

Chemical substances derived from natural products such as plants, animals and microbes have been invaluable as a source of therapeutic agents for all kinds of diseases since the dawn of medicine [1-5]. Even now, natural products still play a dominant role in the discovery of leads to drugs for the treatment of human disease. During the period of 1981 to 2002, of the 877 small molecule New Chemical Entities introduced, nearly half (49%) were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural-product pharmacophores [6], which was defined as an abstract description of the characteristics of a chemical structure that will confer a particular biological activity or the distilled essence of what yields productive ligand-receptor interactions [7, 8]. Traditional Chinese medicines (TCMs), as an important group of the natural products remedies, are gaining increased attention in the drug leads discovery. TCMs have a long history dating back several thousands of years [9]. In China, since about 2700 BC, the TCMs have played the major role in treating diseases. So far, there have been 12,806 medical resources discovered in China, including 11,145 medicinal plants, 1581 medicinal animals and 80 medicinal minerals [10]. The clinic medicinal experience of more than 4000 years and integrated theory system for diagnosis and treatment open a potential shortcut for discovering new bioactive compounds from these natural products.

Screening, analysis and identification of bioactive compounds are always a challenge in natural products research including TCMs. As each TCM raw material may contain hundreds or thousand of components, of which only a few show pharmacological activity [11]. In the past decades, many effective methods have been used for analyzing the components of TCMs in order to screen and identify the bioactive compounds. The routine process for screening is to extract pure compound or single distilled fraction from TCMs, determine its bioactivity by the classic pharmacological means [12-14]. The whole animal model is the most classic pharmacological screening model, which is very important at the aspect of medicine evaluation because it can apparently responses the efficacy, side effect and toxicity of medicines in whole. Although this method is high cost and low efficient, at present it is still a primary way to drug discovery and evaluation. Pharmacological screening with organ and tissue models overcomes some deficiencies of that using animals such as amount of compound needed for screening, but it has rarely been applied in screening of TCMs [15-16]. Cell-based [17-18] screening is rapidly expanding as innovationin target selection and instrumentation increase the number of targets that can be efficiently screened in cellular formats and hence it has become an important means in the screening and evaluation of TCMs.

Molecular screening methods have gained attention because they are rapid, economical, highly sensitive and specific. Many drug targets have been discovered and identified with the development of biotechnology [19, 20]. Despite the complex components and unclear reaction mechanism, the pharmacological effects of the active components in TCMs should also be based on the molecular mechanism. Since Wang *et al*. [21, 22] used receptor and enzyme models for the screening of more than 400 extracts from 150 TCMs, many molecular-based models [23-25] based on the molecular level have been developed for the screening of TCMs for activities.

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In essence, molecular screening is based on the affinity interaction of the bioactive compounds in TCMs with the biomolecular targets. Unlike synthetic drugs, TCMs are often complex natural molecular libraries. Purification of each component is impractical and hence the effective library screening is necessary. With the development in genomic and proteomic technologies and the advances in combinatorial chemistry, recognition studies based on library screening have been applied to the identification of interacting counterparts against known or unknown libraries. A range of small molecule-biomacromolecule interactions have been studied ranging from the chemically simple objects through complex interaction systems [26]. Based on chromatographic and mass spectrometric methods, biological fingerprinting analysis (BFA) was proposed as an approach to investigate the multiple bioactive compounds in natural products. It comprises chromatograms or spectra of the small molecules system carrying with the bioactivity information on the target(s). BFA offers an effective tool for investigation of the small molecule-biomacromolecule interaction in the complex systems and has been used successfully for library screening of TCMs [31, 36, 42, 55-57, 59-67, 72, 73, 75, 89].

2. ADME/TOX PROPERTIES FOR SCREENING OF NATURAL PRODUCTS

Absorption, distribution, metabolism, elimination and toxicity (ADME/Tox) properties of drugs are critical processes in drug discovery and development [27]. In the drug discovery industry, approximately 50% of candidates failed because of poor ADME/Tox properties [28]. To increase the efficiency and reduce the cost of pharmaceutical research, moving ADME/Tox evaluations into early discovery stages has been widely accepted in the pharmaceutical industry [29].

A variety of experimental assays have been developed to characterize each aspect of the ADME/Tox properties. The tools involved include physicochemical methods and biological assays using biomolecules, subcellular fractions, primary cell culture, immortalized cell lines, tissues and whole organs [29]. To be employed in the library screening of the natural products, these assays face the challenge of simultaneous analysis of the multiple compounds, hence the need for efficient biological fingerprinting strategies.

3. CHROMATOGRAPHIC METHODS

As an effective separation tool, chromatography has played a major role in the analysis of the complex systems. In the chromatographic process, analytes are separated on the basis of their different interaction abilities with the stationary phases and mobile phases [30], and TCMs are readily studied in this way. The chromatographic methods, together with the experiment of pharmacology, outline the basic approach to investigate bioactive components in TCMs. However, current chromatographic techniques are still not satisfactory because it is difficult to distinguish the peaks of bioactive components from the other numerous chromatographic peaks, which provides a major bottleneck to the whole screening process, hence the need for biological fingerprinting. The application of biological fingerprinting chromatograms in the natural product screening follows two major strategies. Firstly, the crude extracts are allowed to interact with the target macrobiomolecule prior to the chromatography. Comparison of the target-treated sample chromatograms and the samples that were not treated with the target indicates those components of the extract with the targeted bioactivity. In these strategies, the reversed phase high performance liquid chromatography (RP-HPLC) has been used widely due to its high separation efficiency [16, 31, 36, 42]. In the second approach, the biomolecular target is immobilized as the stationary phase, and chromatographic process directly reflects the interaction of the anlytes and the target on-column [55-57, 59-67, 72, 73, 75].

3.1. Biological Fingerprinting Chromatography by Interaction of Natural Products with Free Target Molecules

BFA, as a tool for screening and analysis of the multiple bioactive compounds in TCMs, was originally defined by our group as the comparison of fingerprinting chromatograms of the extracts of TCMs before and after the interaction with biological systems (DNA, protein, cell, etc.) and was proposed for screening and analysis of the multiple bioactive compounds in TCMs [31]. Microdialysis sampling technique was employed where the small molecules in the samples can be collected for assay, due to the exclusion of large molecules by the microdialysis membrane. The components extracted from TCMs can be simply divided into two groups: some interact with target molecules and are designated as "active components", while others do not. Among the active compounds, those bound to target molecules will be confined in the semipermeable membrane of the microdialysis probe and thus cannot be collected in the microdialysate. Accordingly, in the fingerprinting chromatogram, the peak areas of the interactive components after the interaction have a decrease. While for those not responsible for target binding, because all the molecules can pass through the probe membrane, there is almost no change in their peak areas before and after the interaction. Thus, from the biofingerprinting chromatogram analysis, the target binding compounds in the TCMs extract can be easily distinguished from those not binding.

3.1.1. Targeting DNA

BFA was first applied to the screening of the DNA-binding agents from TCMs. DNA is the molecular target of many antimicrobial, antiviral and antitumour active drugs [32]. And the adduction between genotoxic carcinogens and DNA is believed to be the first step in chemically induced carcinogenesis [33, 34]. Understanding the interaction of drugs with DNA is a necessary first step in elucidating the molecular basis for the potent therapeutic or toxic activities of the compounds. We have analyzed TCMs *Coptis chinensis Franch.* and *Phellodendron amurense Rupr.* on their interaction properties with calf thymus DNA as their biofingerprinting chromatograms shown in Fig. (**1**) [31]. Peak areas showed that seven peaks in *Coptis chinensis Franch.* and three in *Phellodendron amurense Rupr.* decreased after the interaction with DNA, which indicated that they had interaction with DNA. Comparison of retention times and UV spectra with the standards showed that three of them were berberine, jatrorrhizine and palmatine, respectively.

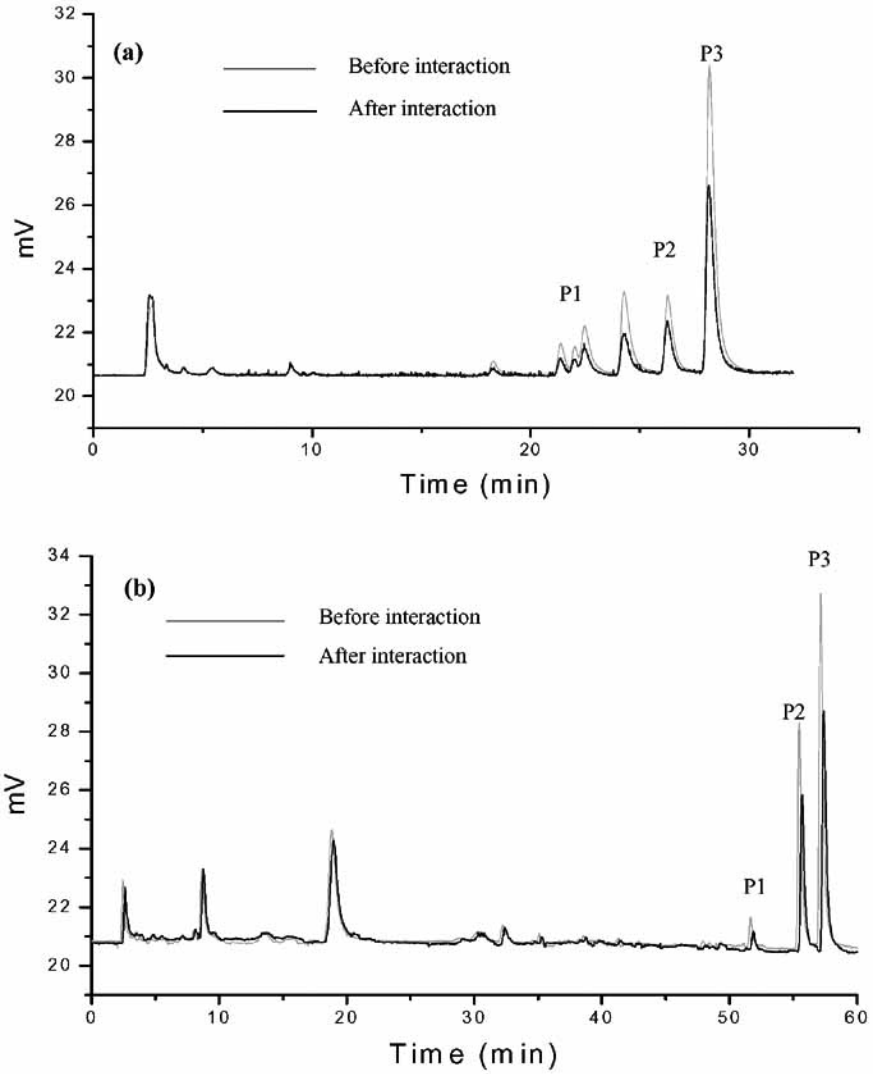


Fig. (1). Biofingerprinting chromatograms for the extract of (a) *Coptis chinensis Franch.* and (b) *Phellodendron amurense Rupr.* before and after the interaction with ct-DNA. Chromatographic conditions: column, 250×4.6 mm I.D. packed with 5 μm Hypersil-BDS; mobile phase, CH_3CN / 20 mM Britton-Robinson buffer (1350 μL H_3PO_4, 1150 μL HAc, 1.236 g H_3BO_3 in 1000 mL water, pH 3.0) containing 5 mM sodium heptenylsulfonate; linear gradient elution, (a) 0 min – 40 min for 20 – 45% CH_3CN; (b) 0−25 min for 11 % CH_3CN, and linear gradient elution, 25−60 min for 15−40 % CH_3CN; ambient temperature; flow rate, 1 mL/min; detection wavelength, 345 nm. Peak identifications: (P1) jatrorrhizine; (P2) palmatine; (P3) berberine.

It was observed that the binding degrees of berberine in the extracts of *Coptis chinensis Franch.* and *Phellodendron amurense Rupr.* were 48.2 and 29.1%, respectively, despite its similar concentrations in the two samples (0.014 mM in the former, 0.015 mM in the latter). As there are many other components present in these extracted samples with variable DNA binding abilities, synergistic or antagonistic effects may influence the binding behaviors of berberine to DNA, which may account for large discrepancies in binding degrees of different extracted samples. This phenomenon is common in the interaction of TCMs complex with the macrobiomolecular target [37].

Changes in DNA structure by the selective binding of small molecules can also play a role in the control of gene expression and may present attractive targets for small molecule therapeutics [35]. On the other hand, knowledge of the structural preferences may also help in understanding the binding mechanisms of small molecules with DNA. To evaluate the DNA structural binding preferences for multiple unidentified compounds in the TCM extracts, centrifugal ultrafiltration sampling has been applied as an alternative to microdialysis. The BFA was improved for the analysis of the samples of low concentration and low volume, and developed for the evaluation of the DNA structural preferences for multiple small molecules simultaneously without purification [36]. In each HPLC run, the binding degrees of every resolved compound in the sample can be obtained simultaneously. The five DNA-binding compounds in the extract of *Coptis chinensis Franch.* and seven *Rheum palmatum L.* were analyzed and each of them was found to has unique structural preference to DNA.

3.1.2. Targeting HSA and Human Serum

Most synthetic drugs show various degrees of binding to plasma proteins [38, 39]. Binding of drugs to plasma proteins, mostly to serum albumin and α_1-acid glycoprotein (AGP), is one of many factors that influences drug distribution [40]. Human serum albumin (HSA) is the most abundant blood plasma protein (~30–50 g/L) and plays an important role in the transport of both endogenous and exogenous compounds such as metabolites, fatty acids, steroids, metal ions and drugs [41]. Reversible noncovalent binding to HSA controls the active concentration of a drug and provides a reservoir for a long-term action, which affects the pharmacokinetic, pharmacodynamic and toxicological properties of a drug.

With the procedure similar to that of DNA, screening targeting HSA has been a necessary step in the TCMs investigation in our lab. HSA binding properties of compounds in hundreds of TCMs crude extracts have been evaluated as well as other characters. The method has also been applied for the screening of bioactive components in TCMs against the whole human blood serum. The biofingerprinting chromatograms of *Rhizoma Chuanxiong* with plasma at pH 7.4 in which some peaks exhibited apparent affinity to proteins in blood serum. Three of them were identified as ferulic acid, 4,5-dihydro-3-butylphthalide (senkyunolide A) and 3-butylphthalide by HPLC-MS and UV spectrum analysis [42].

3.1.3. Targeting Tublin and Microtubules

Microtubules are long, filamentous, tube-shaped protein polymers that are essential components of the cytoskeleton in all eukaryotic cells [43]. Microtubules are extremely important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell into two daughter cells. Thus their importance in mitosis and cell division makes microtubules an important target for anticancer drugs. Microtubules and their dynamics are the targets of a chemically diverse group of antimitotic drugs that have been used with great success in the treatment of cancer [44-46].

Fig. (**2**) shows the biological fingerprinting chromatograms of a kind of *Taxus chinensis* extract targeting microtubules. Comparison of the chromatograms before and after the interaction showed that there were five peaks P1, P2, P3, P4 and P5 had obvious area decrease. With the standard compounds P2, P3 and P5 were identified as baccatin III, cephalomannine and taxol. Among them baccatin III and taxol have been reported as microtubule-binding agents [47]. With the mass spectra, UV spectra and the comparison of the retention times with identified compounds, P1 and P4 were primary identified as 10-Deacetylbaccatin III and 7-epi-10-Deacetyltaxol, respectively. These compounds were promising microtubule-binding agents and their binding properties are under further investigations.

Compared to the traditional pharmacological screening and high throughput screening (HTS), some advantages of the BFA are noticeable in the screening of bioactive compounds from complex natural products. For instance, BFA avoids the most time-consuming step, i.e., purifying pure compound, which is indispensable in the former methods. BFA also combines the biological identification in normal HPLC chromatograms. Such method is applicable to almost

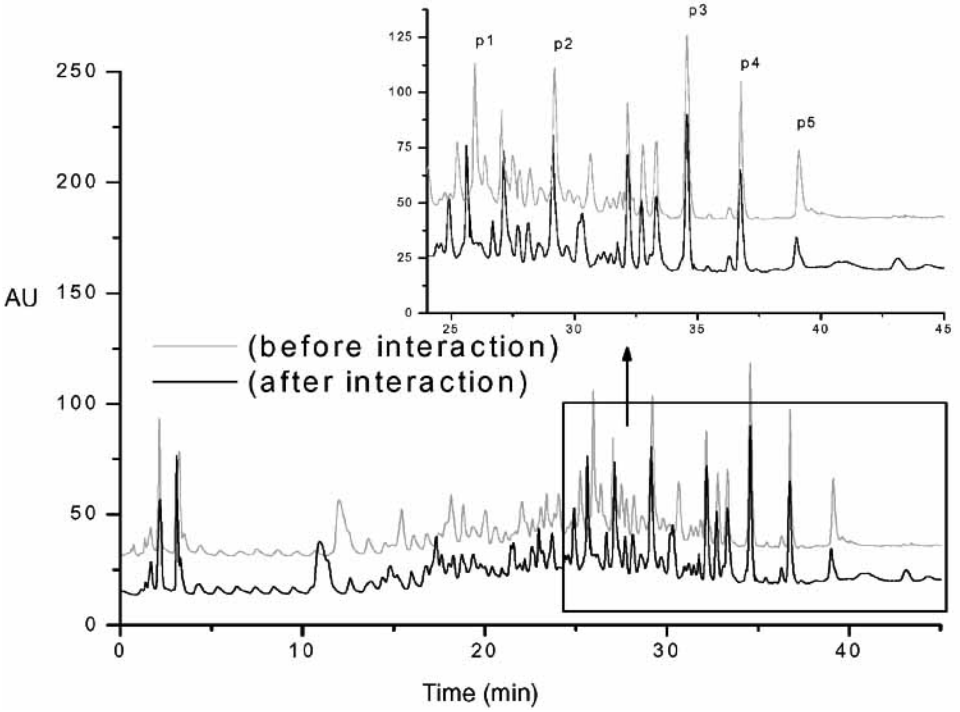


Fig. (2). Biofingerprinting chromatograms for the extract of *Taxus chinensis* before and after the interaction with microtubules. Chromatographic conditions: column, 250×4.6 mm I.D. packed with 5 μm Hypersil-ODS; mobile phase, CH_3CN / H_2O; linear gradient elution, 0 min – 20min – 70 min for 10 – 25 – 95% CH_3CN; ambient temperature; flow rate, 1 mL/min; detection wavelength, 227 nm. Peak identification: (P1):10-Deacetylbaccatin III, (P2): Baccatin III, (P3):Cephalomannine, (P4): 7-epi-10-Deacetyltaxol, (P5): Taxol.

all the targets served in HTS such as enzymes, receptors, DNA and cells.

3.2. Affinity Chromatography with Immobilized Target Molecules

Separation of TCMs by conventional chromatography such as gas chromatography (GC), reversed-phase HPLC (RP-HPLC) and normal-phase HPLC (NP-HPLC) is on the basis of the physicochemical interactions between the analytes and the mobile and stationary phases. Therefore, there is no correlation between their retention and bioactivities. Affinity chromatography is based on the biological interactions between biologically active compounds and immobilized target macromolecules such as proteins, liposome and DNA, and has been successfully applied to rapidly probe drug-target binding and to study anti-cooperative, non-cooperative and cooperative protein–ligand interactions [48-50].

3.2.1. Immobilized Liposome and Biomembrane Chromatography

The activity, toxicity, distribution, and other processes of orally-administered drugs in human body always depend initially on their intestinal absorption across the, epithelial cell membrane. Therefore, the permeability of drugs across biological membrane has been considered as one of most important coefficients to evaluate their bioactivity [51-53].

Liposome can be considered as a type of the artificial membranes (IAMs) mainly formed by phosphatidylcholine in cell membrane and can be noncovalently or covalently immobilized on soft gel particles or silica particles as chromatographic stationary phase to probe the penetration ability of compounds through biological membranes [54-57]. The basis of immobilized liposome chromatography in predicting drug-membrane interaction is the structural similarity between the immobilized phospholipids comprising membrane bilayers [58].

By the immobilized liposome chromatography (ILC), Mao *et al*. [54] found the weighted retention values of drugs under three pHs on the immobilized liposome chromatography showed good correlation with their cell absorption rate constants Papp. Therefore, it can be applied as a model *in vitro* to study absorption and distribution of active components from TCMs in human body. Subsequently, they employed the immobilized liposome on silica gel as the stationary phase of HPLC for the separation and analysis of permeable components in TCMs [55]. More than ten peaks in the extract of TCM *Radix Angelica Sinensis* have significant retention based on their interactions with the ILC stationary phase. Sheng *et al*. [56, 57] expanded the application of the ILC on analysis of the biomembrane permeable compounds to combined prescription of TCMs.

Cell membrane chromatography is another important type of chromatography to simulate membrane absorption. Immobilized biological cell membranes on silica supports can apparently reflect the interaction between analytes and cell membrane combined with the membrane receptors, which is different from the immobilized liposome chromatography. He *et al*. [59,60] firstly introduced the technique for the study of TCMs. This technique has been applied to study the bioactive components of vasodilatation in *Angelica sinensis* [61], *Cladonia alpestris* [62], *Herba eplmedii* [63], *Semen cuscutae* [64] and *Leontice robustum* [65] by immobilized rabbit vascular cell membrane as stationary phase.

3.2.2. Immobilized Plasma Proteins Chromatography

Wang *et al*. [66, 67] first introduced the strategy for screening and analysis of the biologically active components in TCMs with immobilized HSA on silica as the stationary phase. Among the ten major peaks obtained from the methanol extract of *Angelica Sinensis*, two principal biological active components were identified as ferulic acid and ligustilide, which agrees well with the previous reports [68-71]. For another TCM *Artemisia capillaris Thunb*., Wang *et al*. [72] obtained five major peaks and several minor peaks and two of them were identified as scoparone (SCO, 6,7-dimethoxycoumarin) and capillarisin (CAP). In addition, Kong *et al*. [73] resolved the aqueous extracts from four kinds of TCMs on the HSA column and observed that four major active peaks appeared in chromatograms of *Angelica sinensis* and *Radix astragal*, respectively, three in *Rhizoma Chuanxiong* and two in *Paeonia lacti.ora*.

In addition to HSA, AGP is another important plasma protein mainly binding basic drugs [74]. Thus AGP could be used as a complement to serum albumin for the ligand of the stationary phase in affinity chromatography to screen and analyze the active components in TCMs. Wang *et al*. [10] studied the methanol extract from *Radix Salviae Miltiorrhizae* by affinity chromatography with immobilized α-acid glycoprotein as the stationary phase and obtained more than ten peaks; Tanshinone IIA was ultimately identified as one of the principal bioactive components. Moreover, with the same method, in *Rhizoma Chuanxiong*, major peaks and a number of small peaks were resolved based on their affinity to AGP and HSA. Three of them were identified as ferulic acid, chuanxiongzine and ligustilide, all regarded as effective components [75].

3.2.3. Immobilized DNA Chromatography

We developed an immobilized DNA stationary phase on silica for HPLC analysis and bioactive components screening of the TCM extracts. In this approach, genomic DNA was bound onto the surface of amino silica by the formation of a phosphoramidate bonding between the 5' terminal phosphate group and the amino silica. Fig. (**4**) shows a typical chromatogram of the *Coptis chinensis Franch.*on this stationary phase. It can be seen that six main peaks were separated based on their affinity to DNA. By comparison of the retention times and UV/Vis spectra with the standards, one of the peaks was identified as palmatine, another was a combination of berberine and jatrorrhizine. The peak of jatrorrhizine was overlapped by that of berberine due to their great concentration difference. Each of the other four peaks was identified as their corresponding one in the biofingerprinting chromatograms in Fig. (**3**). All the components that interact with DNA were resolved on the immobilized DNA stationary phase.

Application of affinity chromatography to studies of TCMs and natural products has significant advantages. Firstly, the interactions of biologically active compounds with proteins, enzymes and DNA can be probed, even if these com-

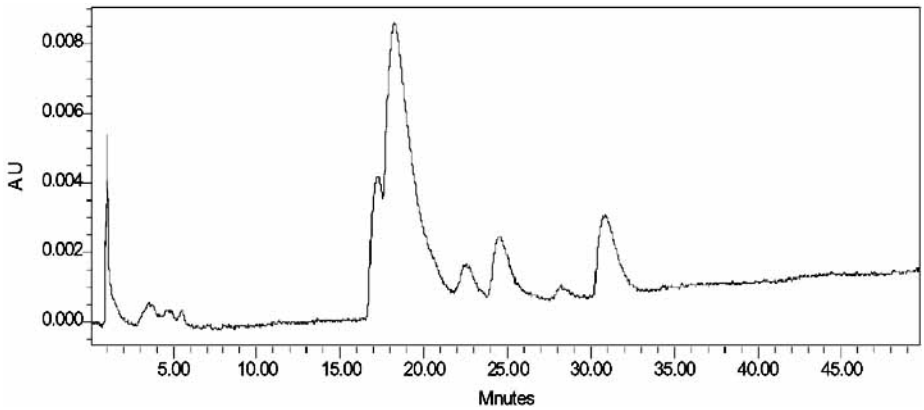


Fig. (3). Chromatograms of *Coptis chinensis Franch.* extract on the immobilized genomic DNA column. Chromatographic conditions: mobile phase, 20 mM tris-HCL buffer (pH 7.4) containing 20 mM NaCl, 5 mM $MgCl_2$, and 2 mM EDTA; flow rate, 1 mL/min; detection wavelength, 345 nm. Peak identifications: (P1) palmatine; (P2) berberine and jatrorrhizine.

pounds have not yet been identified. Secondly, the biologically active components and their biochemical change can be rapidly evaluated and monitored simultaneously. Thirdly, the interactions occurring between biologically active compounds can be studied by adding some effective components screened from TCMs or endogenous compounds to the mobile phase. Therefore, it might be expected that affinity chromatography should play important role in unraveling the mysteries of TCMs.

3.2.4. 2-D HPLC by Coupling Affinity Chromatography to RP-HPLC

Despite the advantages described above, the affinity chromatography may be inadequate due to the low separation efficiency of the biomolecular stationary phase; some peaks can overlap each other and actually represent more than one compound just like the sample above of *Coptis chinensis Franch* on DNA column in Fig. (**3**). Peak overlapping often occurs when the overall number of compounds in a sample exceeds the peak capacity of the column. As mentioned above, the TCMs are very complex samples in themselves, and generally contain up to hundreds or even thousands of different compounds. Therefore, the separation and analysis of components in TCMs become extremely difficult using conventional chromatographic systems. Multidimensional separation techniques provide dramatic improvements in peak capacity. In a two-dimensional (2-D) separation, the total capacity is equal to the product of the peak capacities in

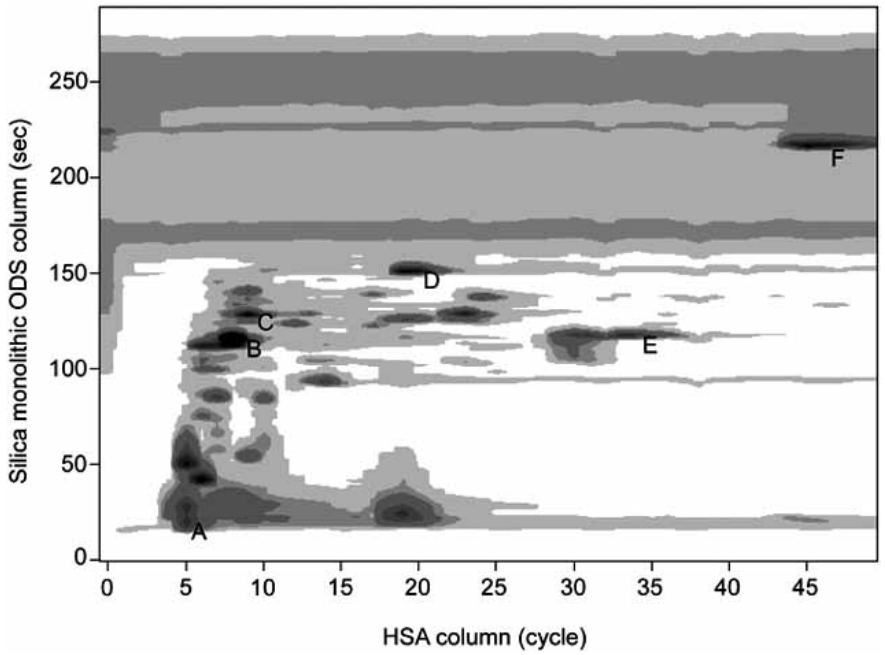


Fig. (4). Two-dimensional chromatogram of *Rheum Palmatum L.* extract. Chromatographic conditions for the HSA column: isocratic elution with 15% acetonitrile in 20 mM ammonium acetate buffer (pH 6.5); flow rate, 0.1 ml/min. Chromatographic conditions for the silica monolithic ODS column: linear gradient elution from 90%B (10%A) to 10%B (90%A) in 3.5 min, and then returning to the initial mobile phase and holding for 1.5 min for re-equilibration; flow rate, 3.0 ml/min; injection volume, 10 μl; detection wavelength, 250 nm.

both dimensions, resulting in much higher resolution. So far, comprehensive two-dimensional liquid chromatography system as a typical form of multidimensional separation system has been widely used to characterize and separate biomolecules, polymers, and other complex mixtures due to its high peak capacity, powerful separation and resolution ability since it appeared in 1990 [76-79]. Our group [80] has built the comprehensive two-dimensional liquid chromatographic separation system for the TCM analysis. The improved 2-D HPLC system by the combination of the affinity column with the immobilized HSA stationary phase and a silica monolithic ODS column was applied to analyze the extract of *Rheum Palmatum L.*[81]. The affinity chromatography with HSA-immobilized stationary phase was applied to separate the bioactive components according to their affinity to protein in the first dimension. Then the unresolved bioactive components retained on the HSA column were further separated on the silica monolithic ODS column in the second dimension. By hyphenating the two-dimensional separation system to DAD and MS detectors, the UV and molecular weight information of the separated compounds were also obtained. Fig. (**4**) is the two-dimensional chromatogram of *Rheum Palmatum L.* extract obtained by this 2D-LC system. It can be seen that in the first ten cycles the spots are thick dotted which means that most components had weaker interactions with the HSA stationary phase and they got little separation on the HSA column. The later eluted components, which exhibits a single peak on the HSA column, also contains several low-abundance components as shown in Fig. (**4**). Because the relative abundance of components in extracts of TCMs usually is usually quite different, some of the peaks were not baseline separated and could not be clearly seen with the blurred spots in the chromatogram in Fig. (**4**). For the presence of high-abundant components and system's background, some of the low-abundance components could not be seen clearly in the two-dimensional plot. Therefore, the chromatograms were manipulated to increase the intensity of the weak signals by normalization of peak heights [82]. Fig. (**5**) is the three-dimensional landscape image of the *Rheum Palmatum L.* extract before and after normalization of peak heights to one eighth of the highest peak height. It can clearly be seen in Fig. (**5**) (b) that more possibly bioactive components can be separated from a single peak from the HSA column after the second dimensional separation by the silica monolithic ODS column and normalization of peak heights. The comprehensive two-dimensional liquid chromatography system herein shows its high peak capacity, sensitivity and powerful resolving potential for biological fingerprinting analysis of the bioactive components in TCMs and natural products.

Rheum Palmatum L. is a commonly used TCM for treatment of hemorrhage of the digestive system, acute hepatitis, gallstone, inflammation of sweat glands and for inhibition of *Helicobacter pylori* [83]. The main bioactive compounds reported are the anthraquinones and their derivatives with a side chain of glucose. By comparing the UV and mass spectra of detected peaks in the *Rheum Palmatum L.* extract with those of standards and reported in literatures [84-86], six of them can be primarily identified as aloe-emodin-8-O-glucopyranoside, chrysophanol-8-O-glucopyranoside, physion-8-O-glucopyranoside, aloe-emodin, emodin-8-O-glucopyranoside and chrysophanol. Their structures and molecular weights were summarized in Fig. (**6**).

3.3. Screening Based on *In Vitro* Metabolism

A drug absorbed orally is transported *via* the portal circulation to the liver, where it is usually subjected to hepatic metabolism followed by elimination as bile or *via* the kidneys. Because the liver is the major organ for drug metabolism, increased throughput screening assays have been developed to determine the metabolic stability of drugs. A method based on HPLC by *in vitro* metabolism with liver homogenate for screening of TCMs was designed by our group [16]. This method can be readily scaled up by an easily controlled process with good analytical reproducibility. Fig. (**7**) shows the fingerprinting chromatogram of TCM *Radix Agelicae sinensis* extract which was incubated with liver homogenate from SD rats. Comparison of chromatograms before and after metabolism. shows that some peaks disappeared and some new ones appeared during incubation. The structural information on these compounds can then be determined primarily by MS analysis. The fraction of peaks was prepared for further structural identification and the efficacy evaluation pharmacological experiments. The metabolites of coniferyl ferulate, ferulic acid ethyl ester and ligustilide were identified using HPLC-MS, UV/Vis and IR. Antineoplastic activity of coniferyl ferulate and ferulic acid ethyl ester was detected by the MTT assay. It was observed that coniferyl ferulate had considerable acute inhibitory activity to HeLa cell culture whereas its metabolite ferulic acid ethyl ester did not show any antineoplastic activity.

4. MS METHODS

4.1. ESI and MS

Electrospray ionization mass spectrometry (ESI-MS) has been widely applied in many investigations of macromolecular interaction with small molecules since they were introduced into the mass spectrometer directly from solution. When used in natural products analysis, ESI-MS rapidly evolved to powerfully complement of other HPLC detection systems. As a result, the interface of HPLC with ESI-MS has provided a method that has effectively facilitated the integration of complex mixtures with the rapid drug discovery processes. Some strategies [87-92] were developed using ESI-MS method for the natural products screening. De Boer *et al*. [89] screened enzyme inhibitors by an ODS column coupling to a continuous-flow enzymatic assay with ESI-MS as readout. Using cathepsin B as the model enzyme, three compounds from red clover (*Trifolium pratense L.*) extract showed their binding activity. Hofstadler *et al*. [90] evaluated the affinity of more than 67 000 putative ligand substrate pairs in a 24 h screening run by multiplexing both targets and compound collections based on the electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR) method. After that, this method was also used to evaluate the noncovalent interactions between multiple RNA-based drug targets and components derived from a bacterial natural product library [87]. In the near future, the ESI-MS can be expected to apply in BFA of the TCMs targeting their ADME/Tox properties.

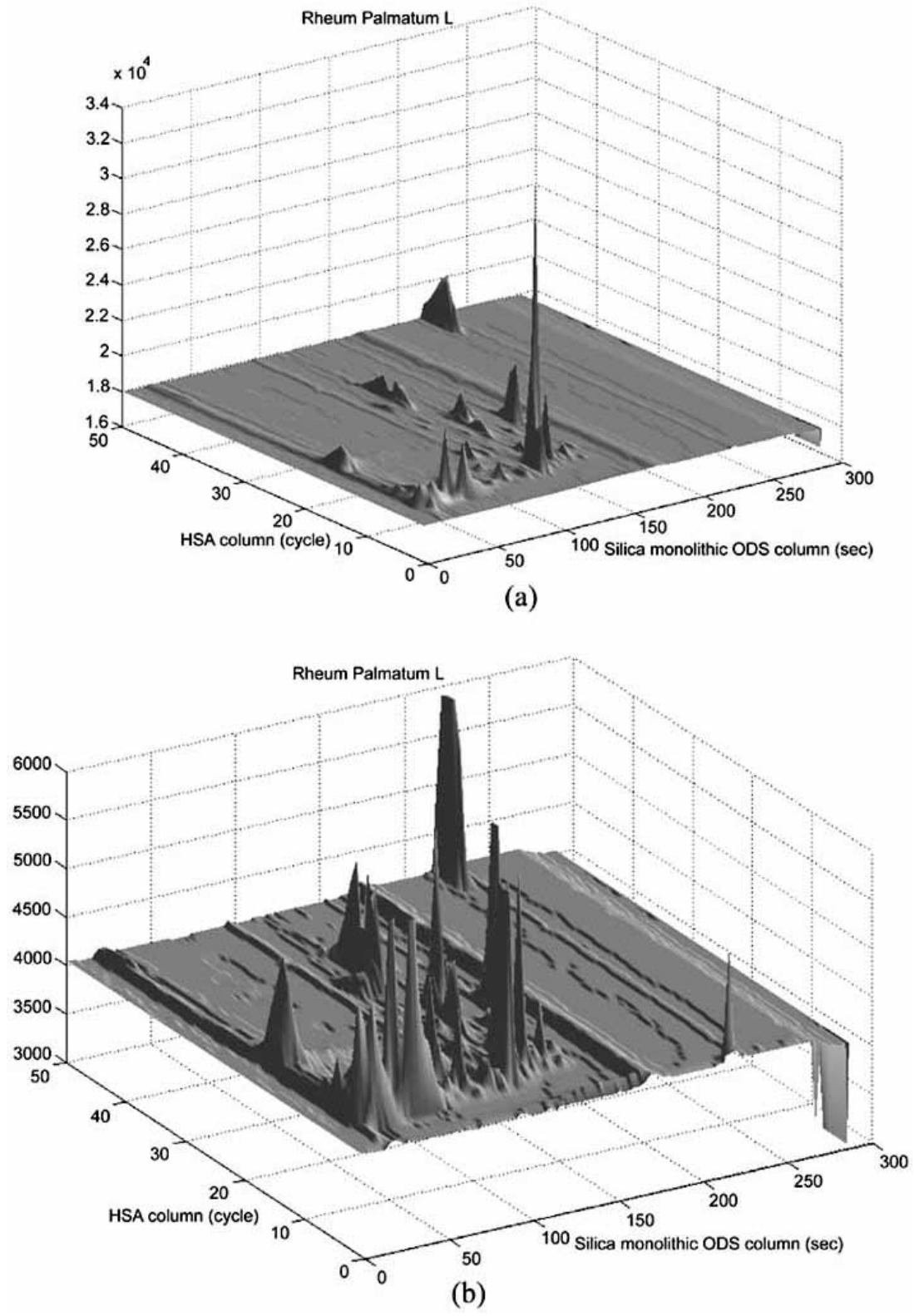
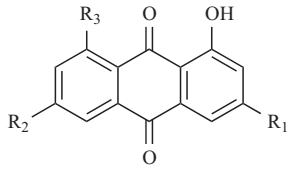


Fig. (5). Three-dimensional landscape images of *Rheum Palmatum L.* (a) The original image without normalization. (b) After normalization by a value of one-eighth of the highest peak. Chromatographic conditions for the HSA column: isocratic elution with 15% acetonitrile in 20 mM ammonium acetate buffer (pH 6.5); flow rate, 0.1 ml/min. Chromatographic conditions for the silica monolithic ODS column: linear gradient elution from 90%B (10%A) to 10%B (90%A) in 3.5 min, and then returning to the initial mobile phase and holding for 1.5 min for re-equilibration; flow rate, 3.0 ml/min; injection volume, 10 μl; detection wavelength, 250 nm.



R_1	R_2	R_3	Compound
CH_3	H	OH	Chrysophanol (MW254.23)
CH_2OH	H	OH	Aloe-emodin (MW270.23)
CH_3	H	O-$C_6H_{11}O_5$	Chrysophanol-8-O-glucopyranoside (MW416.38)
CH_2OH	H	O-$C_6H_{11}O_5$	Aloe-emodin-8-O-glucopyranoside (MW 432.38)
CH_3	OH	O-$C_6H_{11}O_5$	Emodin-8-O-glucopyranoside (MW 432.38)
CH_3	OCH_3	O-$C_6H_{11}O_5$	Physion-8-O-glucopyranoside (MW 446.41)

Fig. (6). The molecular structures and weights of the identified compounds in extract of *Rheum Palmatum L.*

4.2. MALDI-TOF-MS

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) has been developed to analyze small molecules successfully by using the different matrix substances, such as desorption/ionization on porous silicon (DIOS), matrix with high molecular weight and surfactant suppressed matrix and inorganic materials. DIOS technique has been employed to detect small molecules bound to protein immobilized on porous silicon [93]. This technique has also been used to probe the relative binding affinity of different ligands to a target protein. It was also used in the protein identification and protein inhibitors screening through in-situ digestion with immobilized proteolytic enzyme on the porous silicon surface [94]. Due to its high sensitivity, fast analysis, low consumption of sample and easy automation, this technique allows the screening of drug leads in complex system such as natural products. With the protein immobilized DIOS technique, screening of the

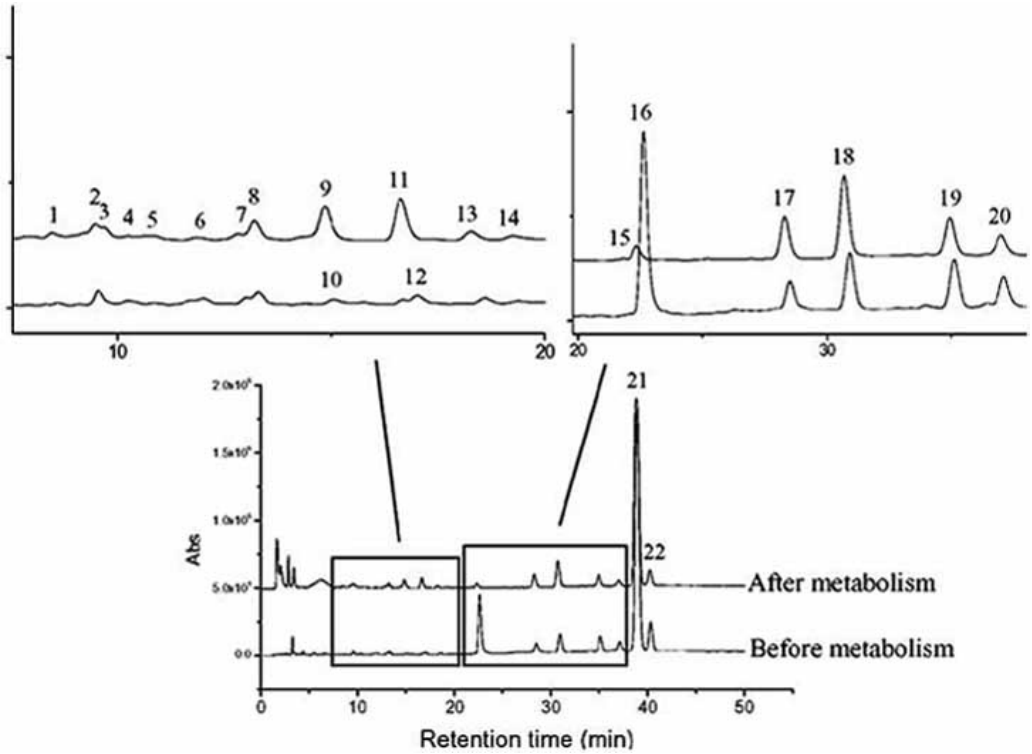


Fig. (7). Biological fingerprinting chromatograms for extract of *Radix Agelicae sinensis* before and after *in vitro* metabolism with SD rat liver homogenate. Chromatographic conditions: column, 200×4.0 mm I.D. packed with Kromasil C_{18} (5μm). Mobile phase, the elution was performed with the linear gradient from 20% to 60% acetonitrile/water (v/v) in 50 min. Flow rate was 0.76 mL/min, and the UV detection wavelength was 205 nm. Peak 9, ferulic acid ethyl ester; peak 16, coniferyl ferulate; peak 21, ligustilide.

binding agent in the TCM extracts has been realized by MALDI-TOF-MS.

Pan *et al*. [95] developed oxidized carbon nanotubes as a matrix of MALDI-TOF-MS for analysis of small molecules. Reliable quantitative analysis of jatrorrhizine and palmatine with a wide linear range and good reproducibility of relative peak areas was achieved using this matrix. This matrix was adapted in our lab for the BFA of several TCMs. Fig. (**8**) shows fingerprinting spectra comparison of TCM *Radix Agelicae sinensis* before and after metabolism with rate liver homogenate *in vitro*. As in the fingerprinting chromatogram obtained by HPLC, some peaks disappeared and some new ones appeared after metabolism. These peaks can be primarily identified by their molecular weight. The change of the concentration of each compound can also be obtained from the quantitative analysis. The fingerprinting spectra gave the metabolic information according to the *m/z* of the compounds. Due to the different selectivity and sensitivity, the result obtained by the MS analysis is a complement to chromatographic methods.

5. CONCLUSIONS AND PERSPECTIVES

The screening and analysis of the multiple bioactive compounds in complex system of TCMs are a formidable challenge. BFA, on the basis of the chromatographic and MS methods, provides a valuable alternative for this task. The comparison of the chromatograms before and after the interaction with the free targets can distinguish the active ones quickly from the large number of compounds in the TCMs. Affinity chromatography simulates the interaction of the multiple small molecules with the immobilized biomolecules; while through the 2-D HPLC by coupling biochromatography to RP-HPLC, a higher resolution can be obtained. ESI-MS is a powerful complement to other HPLC detection methods. The strategies on the basis of both HPLC-ESI-MS and MALDI-TOF-MS can be expected to play more important roles in the BFA in the future. The BFA displays several advantages in library screening including the direct analysis of crude extracts of the natural products without further purification of each compound, which is the most time-consuming step in the traditional screening methods. Besides, biological interaction information can be deduced from the chromatograms or spectra, from which a certain compound can be estimated to be active or inactive and the relative binding ability of the active ones can also be obtained. Finally, the approach has a wide applicability to the cases of nearly all the interaction systems of small molecules and macromolecules. Besides the ADME properties, the application of the BFA can be extended to the other screening targets. In addition, it is fast, simple, of high efficiency and high throughput.

Because of the great need in biological research and drug discovery, the drug screening system is becoming increasingly complex, from single drug-single target to drug library-target, even to the drug library-target library. Therefore, more rapid and more efficient methods are required to directly obtain binding information from multiple systems. Thus BFA shows great promise for future development as a drug discovery tool in library research.

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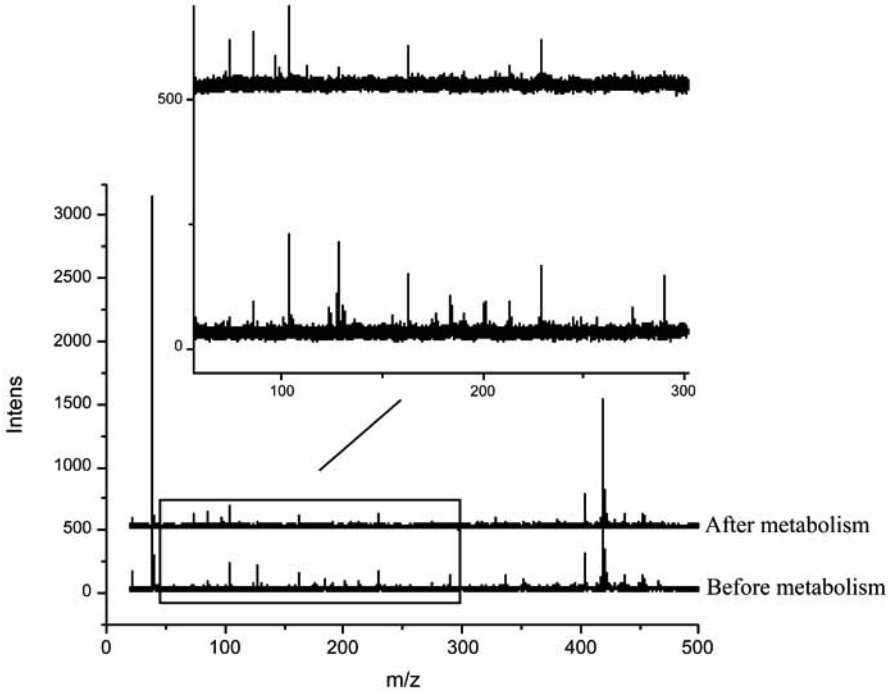

Fig. (8). MALDI-TOF-MS fingerprinting spectra for the extract of *Radix Agelicae sinensis* before and after metabolism with SD rat liver homogenate.

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