Application of Chromatography Technology in the Separation of Active Components from Nature Derived Drugs

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Abstract: Chromatography technology has been widely applied in various aspects of the pharmacy research on traditional Chinese medicine (TCM). This paper reviews literatures, published in the past decades, on the separation of active component from TCM using chromatography technology. Ultra-performance liquid chromatography (UPLC), high-speed counter-current chromatography (HSCCC), rapid resolution liquid chromatography (RRLC), supercritical fluid chromatography (SFC), affinity chromatography (AC), and bio-chromatography (BC) are introduced in detail. Compared to high performance of high-performance liquid chromatography (HPLC), analysis time and solvent loss are significantly reduced by UPLC with increase in resolution and sensitivity. Some ingredients from nature derived drugs can be separated more completely by HSCCC, which has remarkable characteristics such as low cost, simple operation and no pollution. Trace components from complex systems can be selectively and efficiently separated and purified by AC, This feature makes it effective in isolation and identification of active components of Chinese herbs. Interference of some impurities could be excluded by BC. Active ingredients that are difficult to be separated by normal method can be acquired by SFC. Currently, application of novel chromatography techniques in TCM is still in the exploratory stage and many problems, such as preparation of stationary phase and detection, need to be solved.

Keyword: Nature derived drugs, traditional Chinese medicine (TCM), chromatography technology, bioactive compound, separation.

1. INTRODUCTION

Active ingredients from nature derived drugs have been the foundation for treatment of diseases for thousands of years in China [1]. Traditional Chinese medicine (TCM), generally made by Chinese herbal medicine mixture and a series of physical and chemical reactions, is characterized for its complex composition, low efficacy and toxic and side-effect. Therefore, how to efficiently analyze active ingredients of this complicated and undefined system to enhance efficacy and reduce toxicity of the TCM has become a sticking point in the clinical application and development of TCM [2]. However, the separation of active ingredients is a difficult problem for the analysis of active components. Hence the separation and identification of the active ingredients in nature derived drugs have become urgent.

Traditionally, there are many separation methods to separate some active ingredients from TCM, including system solvent, two-phase solvent extraction, salt-out method, fractionation, crystallization, etc., but separation efficiency of these methods is too low [3]. Extraction procedures are either efficient, or the extracts are not safe for human consumption due to potential toxic residues [4].

In recent years, novel separation technologies with high efficiency are gradually applied in the extraction and separation of active ingredients [1]. These techniques include macroporous resins, membrane, molecular distillation technology, microwave [3], supercritical fluids extraction [5], ultra-filtration [6] and chromatography technology, etc., among which chromatography technology has been widely applied in the research fields of petroleum, chemistry, chemical engineering, biomedicine, and food. Large-scale purification of natural compounds by chromatography technology in medical, biological, and fine chemical industries increases greatly There are many compounds of pharmacological interest has been studied by chromatography technology [7, 8].

At the beginning of last centaury, Russian botanist Tswett put forward the concept of chromatography and recognized its potential as a separation technology, when he researched the composition of plant pigment [9, 10]. The features of chromatography, high resolution, rapid and low cost, accurate screening [11], attracted a growing number of researchers. Its main advantage lies in excellent performance of separating target ingredients from extremely complex compounds [12]. Chromatography meets the need of acquiring high purity ingredients from complex system high efficiently and sensitively [13]. Application and development of chromatography technology play a significant role in promoting the research on TCM. This paper gives an overview on the research progress of several important chromatography, ultra-performance liquid chromatography (UPLC), high-speed counter-current chromatography (HSCCC), rapid resolution liquid chromatography (RRLC), supercritical fluid chromatography (SFC), affinity chromatography (AC), and bio-chromatography (BC), used for separation of active ingredients from TCM in recent years.

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2. LIQUID CHROMATOGRAPHY (LC)

2.1. UPLC

UPLC, based on well-established principles of LC technology, is a new separation technique, which utilizes 1.7 μm particles as stationary phase and was introduced by the Waters company in 2004 [14]. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis [11]. Because of system performance of UPLC over conventional high-performance liquid chromatography (HPLC) with respect to analysis time, efficiency, sensitivity, capacity of peak, UPLC get the further extension and expansion. It improved extremely the quality and efficiency of analysis, which provides a platform for the separation and analysis of complex system.

The quality control analysis of four TCM pharmaceutical formulations were transferred from HPLC to UPLC system with respect to number of theoretical plates, symmetry factor, resolution, reproducibility, researched by Nov′akov′a *et al*. [13]. The results showed that the transformation from HPLC to UPLC is feasible and simple. There are more obvious advantages for UPLC in terms of analysis time, resolution, sensitivity. UPLC is more applicable for analysis of pharmaceutical complex samples [15]. Because of its extremely short equilibrium and analysis time, and low solvent lost in gradient elution chromatography experiments, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis [16].

For example, alkaloid is well separated with UPLC [17] only by adding acid inhibitor into the mobile phase rather than adding amines as common HPLC. Analysis of complex pharmaceutical system and separation of some active ingredients will start a new phase for its ultra-analysis-speed and ultra-sensitivity. Recently, some new results are obtained by UPLC in separation of active ingredients from some TCM (Table **1**).

2.2. RRLC

RRLC, a new category of separation technique with sub-2um filler, has finer granularity, shorter column diameter and longer column length compared with conventional column. Therefore, it can achieve high the speed of separation and analysis within shorter equilibrium and analysis time. Yoshida *et al*. [18] isolated tea polyphenols from green tea by this technique. The results showed that transformation from HPLC to RRLC is feasible, and the separation speed is 15 times shorter than HPLC under the same condition of resolution and purification.

In addition the major bioactive compounds flavonoids isolated from *Fructus Aurantii Immaturus* by RRLC. The main components of flaconoids were hesperidin, naringin, neohesperidin and naringin in 19 of 27 batch samples [19]. According to the content of each component in these samples, they were divided into two groups: one was high in the contents of hesperidin and naringin, and the other was rich in contents of neohesperidin and naringin. The main components of flaconoids were hesperidin and naringin in the rest batches with absence of neohesperidin and naringin. The total content of flaconoids in all batch samples was about 12.4~38.7 %. The analysis time of RRLC was less one third in contrast to HPLC under the same conditions of resolution, sensitivity, and precision. This method not only shortened the analysis time, but also reduced the solvent consumption and environmental pollution. Although relatively little research was done in this subject, it has been successfully utilized for detecting the content of active ingredients from TCM (Table **1**).

2.3. HSCCC

HSCCC, with solvent system consisted of incompatible two or three phase solvent, is a novel liquid-liquid separation technology to separate multiple components with a wide range of hydrophobicity. HSCCC with three phase solvent system consisted of *n*-hexane– methylacetate–acetonitrile–water (4:4:3:4, v/v/v/v) has been proved to be a more efficient preparative technique in dissociating of active components from TCM [28]. In terms of different capacities of distribution in two or three phase solvent, transfer speed is also varied. In consequence each ingredient is separated more completely [29].

Certainly HSCCC has many advantages: 1) Liquid stationary phase, which makes up the defect of time-consuming of common countercurrent chromatograph, is fixed by centrifugal effect. 2) Stationary phase, with no vector, eliminates the adsorption phenomenon of gas chromatography (GC) with vector, which is especially applicable for preparative separation. 3) Solvent system is not only served as both stationary and mobile phases, but is cheap and easy to obtain. It can be changed at any time without special requirements. Savitri Kumara *et al*. [30] testified that proanthocyanidins from tea were purified more completely by HSCCC with solvent system of *n*-hexane–EtOAc–MeOH–water (1:5:1:5). 4) A large quantity of sample could be acquired in shorter time, and there was no pollution for chromatography column. 5) It is reliable, fast and accurate, low-cost and simple to operate in analysis and separation.

Compared with the separation performance of atmospheric pressure and low pressure chromatography, isolation ability of HSCCC is better. One or more monomer of high purity can be separated from some samples for just one time by HSCCC, and generally whole process of separation can be finished in several hours [31]. Feng [32] employed HSCCC for the first time to separate the flavonoids from *Trollius Chinensis Bunge* and successfully acquired one pure chemical composition identified as vitxin. The results obtained by HSCCC in recent years are show in Table **2**.

3. SFC

SFC is a method employing supercritical fluid (SF) as mobile phase, and solid sorbent or polymer bonding into vector as stationary phase. Compared to LC, features of SFC are the lower operating temperatures, the higher diffusivities of the solutes, and the lower viscosity of the eluent [49], which provides many advantages such as shorter analysis time, yielding at least a three-fold increase in the throughput, easy recovery of products by simple decompression, low consumption of organic solvents and wider range of applicability [50, 51]. SFC has more advantages in separating

Table 1. Application of UPLC and RRLC for Separation and Detection of Active Ingredients from Some of TCM

Herbs	Active ingredients	Chromatography Technology and column	Gradient condition	Mobile phases; the low rate; temprture	Results	Refs.
Fructus aurantii	polymethoxylated flavones (PMFs)	UPLC; ACQUITY UPLCTM BEHC18 (Waters, Milford, USA), 100 mm×2.1 mm×1.7 μm	0~13 min., 50 % B; 13~18 min., 50~65 % B; 18~20 min., 65 % B	A: water/formic acid (v:v, 100/0.1); phase: methanol/formic acid (v:v, 100/0.1); 0.25 ml/min.; 30 °C	The method was validated by tangeretin and sinensetin, two representative compounds, PMFs, and 44 PMFs, were separated and identified.	[20]
Epimedium	flavonoids	UPLC; BEH C_{18}(50 mm×2.1 mm I.D., 1.7 μm)	0~2 min., 20~24 % B; 2~4 min., 24~26 % B; 4~5 min., 26~32 % B; 5~12 min., 32~35 % B; 12~15 min., 35~100 % B; and finally, reconditioning the column with 20 % B isocratic for 3 min. after washing column with 100 % B for 2 min.	consisting of water with 50mM acetic acid (A) and acetonitrile (B); 0.25 ml/min.; 25 °C	The R.S.D.s for intra- and interday of 15 analytes were less than 5.0 % at three levels, and the recoveries were 95.0~103.7 %. The validated method was successfully applied to quantitatively analyze 15 flavonoids in different species of *Epimedium*.	[21]
Panax notoginseng	11 kinds of saponins containing Notoginsenoside R1	UPLC, BEH C_{18} column (50 mm×2.1 mm I.D., 1.7 μm)	0~5.5 min., 18~19 % B; 5.5~6.0 min., 19~31 % B; 6.0~9.5 min., 31~35 % B; 9.5~12.0 min., 35~56 % B, and finally, reconditioning the column with 18 % B isocratic for 2 min. after washing column with 100 % B for 3 min..	water (A) and acetonitrile (B); 0.35 ml /min; 45 ◦C	The overall intra- and inter-day variations (R.S.D.) of 11 saponins were lower than 3.1 %.and overall recovery of 93.0~101.6 % for the analysis. Analysis time reduced 4/5 compared to HPLC.	[22]
Tea	flavonoids phenol	UPLC; BEH C_{18} analytical column(100 mm × 2.1 mm, 1.7 μm, Waters, Czech Republic)	0~4 min., 88.5 % A, 11.5 % B; 4~20 min., linear gradient elution from 88.5 % A, 11.5 % B to 50 % A, 50 % B.	0.1 % formic acid (buffer A) and methanol (buffer B); 0.45 ml/min.~0.42 ml/min., 25 °C	UPLC was effective method for the identification and quantification of phenolic compounds and caffeine.	[23]
Cordyceps	14 nucleosides and nucleobases	UPLC; BEH C18 column (50 mm×2.1 mm I.D. particle size, 1.7 μm)	0~3 min., 0~3 % B; 3~10 min., 3~20 % B; and finally, reconditioning the column with 100 % A isocratic for 3 min. after washing column with 50 % B for 3 min..	0.5 mM acetic acid (A) and acetonitrile (B); 0.25 ml/min; 25 °C	The developed method was applied for the analysis of nucleosides and nucleobases.	[24]
Si-Ni-Decoction	Aconitine, gingerols, triterpenoid saponin, flavonoids	RRLC; SH ISEDO APCELL PAK C_{18}(50 mm×2.0 mm, 2 μm)	0~5min., 5~15 % B; 5~10min., 15~22 % B; 10~22 min., 22~30 % B; 22~35 min., 30~40 % B; 35~40 min., 40~60 % B; 40~43 min., 60~65 % B; 43~45 min., 65 % B	water containing 0.1 % formic acid (A), acetonitrile(B); 0.25 ml/min.; 25 °C	Established an efficient and rapid method to identify and separate the effective composition of Si-Ni-Decoction.	[25]
Panax ginseng	Ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1	RRLC; ZOBAX SB-C_{18} column (3.0 mm×50 mm, 1.8 μm)	0~14 min., 19 % A; 14~24min., 19 ~34 % A; 24~36 min., 36 % A	acetonitrile(A), water(B); 1.0 ml/min.; 35 °C	This method showed the accurate and good repeatable characteristics in determination of active ingredients from *Ginsng*.	[26]
the root of *Bupleurum*	saikosaponins	RRLC; Zorbax SB-C_{18} column (1.8 μm, 3.0 mm×50 mm I.D., Agilent Technologies, USA)	0–6.5 min., 30~40 % A; 6.5~9 min., 40~50 % A; 9~10 min., 50~100 % A, 10~12 min., 100~100 % A	acetonitrile (A) and water (B); 0.8 ml/min.; 25 °C	The analytical method is highly effective for the quality evaluation of *Bupleurum* species.	[27]

Table 2. Application of HSCCC for Separation of Active Ingredients from TCM

herbs	Solvent systems	Active ingredients	The results	References
flavonoids				
Aloe vera	chloroform-ethanol-water (4: 3: 2); dichloromethane-methanol-water (5: 4: 2)	cinnamoyl-C-glucoside aloe chromone	A purity of more than 95 % for cinnamoyl-C-glucoside aloe chromone.	[33]
Extract of *Ginkgo Biloba*	chloroform-ethanol-water (4: 3: 2); dichloromethane-methanol-water (5: 4: 2)	bilobalide and hesperidin	Pure hesperidin and stationary phase retention of 79 % were acquired using HSCCC for the first time.	[34]
Hippophae rhamnoides	chloroform-ethanol-water (4: 3: 2); dichloromethane-methanol-water (5: 4: 2)	isorhamnetin	Isorhamnetin was well separated from kaempferol and lipophilic impurities.	[35]
Patrinia villosa Juss	hexane-ethyl acetate-methanol-water (10: 11: 11: 8)	bolusanthol B,5,7,2′,6′-tetrahydroxy- 6,8 - 2(γ,γ – dimethyl allyl) flavanone and tetrap terol.	Bolusanthol B and tetrapterol I were obtained from the plant of *Patrinia* genius for the first time; flavanone was a novel prenylated flavonoid and discovered from nature for the first time.	[36]
Astragalus membranaceus Bge. var. mongholicus (Bge.) Hsiao	hexane-chloroform-methanol-water (1.5: 3: 3: 2)	calycosin and formononetin	A purity of calycosin is more than 95 %.	[37]
The seeds of *Oroxylum indicum*	hexane-ethyl acetate–methanol- water (10: 11: 11: 8)	three flavonoids	A purity of calycosin is more than 95 %.	[38]
leaves of *Ampelopsis grossedentata*	hexane- ethyl acetate –methanol-water (1: 3: 2: 4)	(+)-dihydromyricetin	The (+)-dihydromyricetin was obtained at a high purity of over 99 %.	[39]
Alkaloids				
The root of *Aconitum coreanum*	chloroform-methanol-0.2mol/l Hydrochloride (10: 3: 3)	guanfu base R,guanfu base G, guanfu H, guanfu base Z, guanfu base A, *et al*.	Eight components contents Guanfu base R, were obtained once, and its structure is 2α-propionyl-11α,13β-diacetyl-14-hydroxyhetisine.	[40]
Aconitum carmichaeli	chloroform-methanol-0.3mol/l Hydrochloride (4: 3: 2)	15-α-hydroxy new ukrainian monomer	15-α-hydroxy, new Ukrainian monomer, was separated successfully by HSCCC for the first time.	[41]
Tinospora capillipes gagnep	chloroform-methanol-0.2mol/l Hydrochloride (2: 1: 1)	palmatine	A purity of Palmatine is more than 95 %.	[42]
Organic acids				
Grape pomace	hexane- ethyl acetate –methanol-water (3: 7: 3: 7)	hydroxy cinnamoyltartaric acids	A purity of 97.0 % for caftaric acid; A purity of 97.2 %for coutaric acid; A purity of 90.4 % for fertaric acid.	[43]
Salvia miltiorrhiza	hexane- ethyl acetate –water - methanol (1.5: 5: 5: 1.5)	Salvianolic acid B	A purity of 98.6 % for Salvianolic acid B.	[44]
Flos lonicerae	butanol- glacial acetic acid- water(4: 1: 5)	chlorgenic acid	purity of chlorogenic acid over 94.8 % with approximately 90 % recovery.	[45]

(Table 2). Contd…..

herbs	Solvent systems	Active ingredients	The results	References
Others				
Aloe vera extract	chlorform- methanol-acetone-water (9: 8: 1: 8)	aloin and aloe-emodin	A purity of 98.87 % for Aloin; A purity of 95.66 % for aloe -emodin.	[46]
Aloe crude extract	chlorform-methanol-water (9: 10.5: 8)	aloin and aloe-emodin	Components were obtained once .A purity of 99.99 % for aloin and a purity of 95.83 % for aloe-emodin.	[47]
Hilberry fruit crude extract	methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1: 4: 1: 5:0: 0.1, v/v)	anthocyanins	Valuable attributes of separating anthocyanins by HSCCC compared to preparative HPLC are high sample loading capacity, no irreversible adsorption effects of analysis to solid-phase column material, complete sample recovery and the much lower mobile phase usage.	[48]

antibacterial medicine under the condition of higher resolution and shorter analysis time compared to HPLC. According to the characters of the mobile phase of HPLC, HPLC can be transferred easily to SFC, which can be used for the analysis of some thermo-sensitive, volatile and polarity components [52]. Currently schematic diagram of SFC used for separation of active ingredients from TCM shows as Fig. (**1**). Because of its special character, SFC has been applied in sepa-

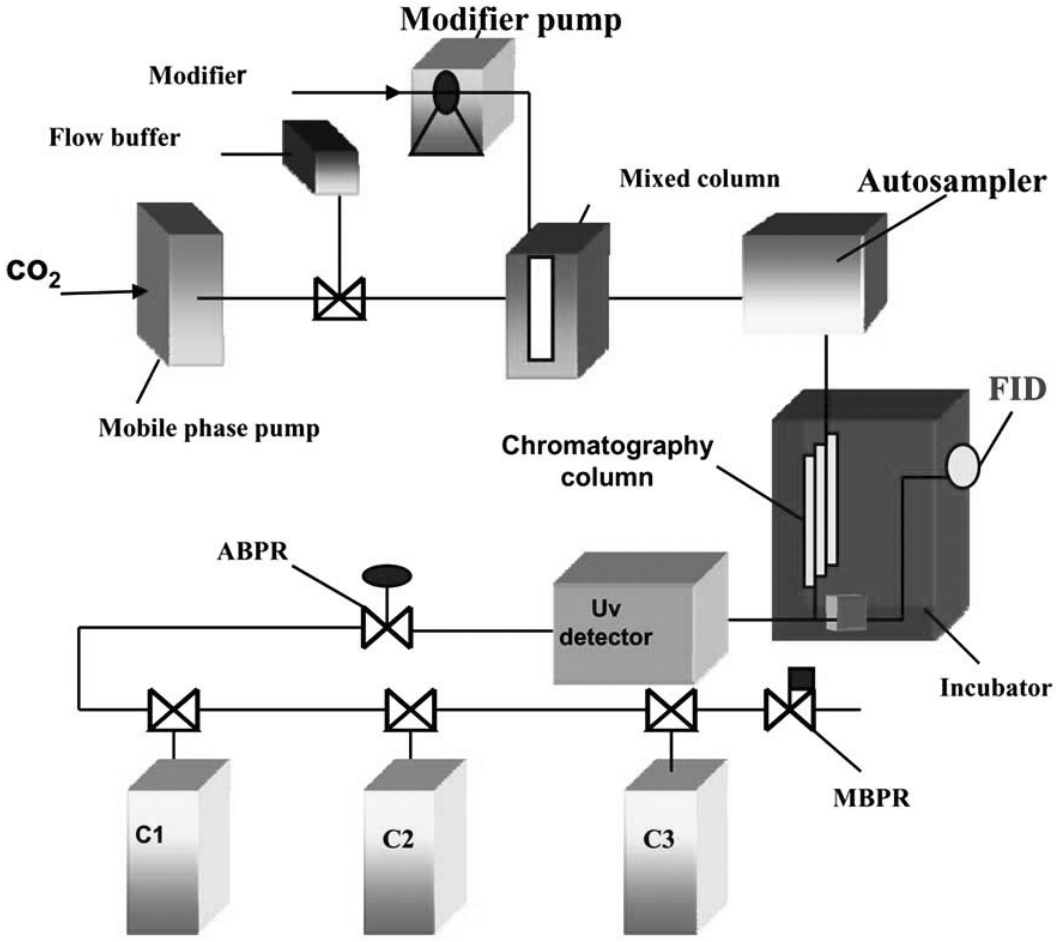

Fig. (1). Schematic diagram of the SFC used in the present study. ABPR: back pressure regulator; C1, C2 and C3: cyclonic separators; MBPR: manual restrictor. The CO_2 and modifier are pumped by high pressure pumps, and the CO_2 pump is cooled by a circulating bath at 5 °C. The column pressure is controlled by a back pressure regulator and the column is coupled to an UV/vis detector. The range of column pressures/temperatures tested was from 80 to 200 bar, and from 40 to 80 °C. The CO_2 flow rate was kept constant at 20 g/min. Ethanol was used as a modifier at different percentages between 5 and 15 %.

ration of active ingredients from TCM in an increasingly wide range [53, 54].

Parthenolide in wild *Chamomile* was separated and analyzed using SFC [54]. The precision and linearity of the detector response were excellent. Under the condition that the extracts of wild *Chamomile* were not gotten any treatment, it was found that, the determination of the sample could be finished just within 3 min. Compared with GC and HPLC, analysis time was greatly shortened but the same separation performance with GC was obtained.

Dauricinoline and daurinoline from the peel of a species of *Zanthoxylum schinifolium* Sieb et Zucc were successfully separated by SFC. Two isomers of alkaloids from *Menispermum* root, nuolin alkali and genohrin alkali, were acquired successfully by the preparative-supercritical fluid chromatography (Prep-SFC) [55].

4. AC

AC is a specific and effective chromatography technique *via* using or imitating reversible interactions between biomolecules to extract, separate, and analyze certain matter from complex samples [56-57].

4.1. Molecular Imprinting

Molecular imprinting, based on molecular interaction, is very old but its applications in various fields are emerging recently. It consists of polymerizing a functional monomer, mixed with a template, in the presence of a cross-linking agent. Removal of the template leaves an imprinted cavity in the polymer, which provides a selective binding site for this template [58]. The initial examples of molecular imprinting by the use of synthetic organic polymers and have since then found applications in separation processes (chromatography, capillary electrophoresis, solid phase extraction, membrane separation), microreactors, immunoassays and antibody mimics, catalysis and artificial enzymes, biosensor recognition elements and bio- and chemo-sensors [59-61]. Currently, AC with molecular imprinting polymer as stationary phase is feasible for identification and separation of active components from TCM.

Harmaline and harmine existing in *Peganum nigellastrum* show antitumor activity. An imprinted polymer, with two harman structural analogues as template, has high affinity for harmaline and harmine. Harmaline and harmine in the crude extract of *Peganum nigellastrum* were separated and identified simultaneously from the complex matrix by connecting the molecular imprinting affinity column with a positive atmospheric pressure chemical ionization (API) time-of-flight (TOF) mass spectrometry (MS) [62].

Using acrylamide as functional monomer and quercetin, strong polar compounds, as template, a molecular imprinted polymer was prepared in polar solvents by non-covalent method. Liquid chromatography experiments show that the polymer has a specific affinity with quercetin and can be directly used to separate compounds from hydrolyzate of biloba leaves. Two flavonoid components, quercetin and its structural analogue kaempferol were obtained [63].

4.2. Immunoaffinity Chromatography (IAC)

IAC is a method using the inter-reversible binding role of antigen and antibody to selectively separate and purify trace components from complex systems. Antibody is fixed on the solid phase vector, thus target components from biological, pharmaceutical, food or environmental samples can be separated effectively by IAC [64, 65].

In order to deplete the component naringin from Si-Ni-San (a traditional Chinese prescription), an IAC column with antibody of anti-naringin was prepared. The results showed that, except the peak of naringin, other peaks have no obvious changes. By using this column, naringin in Si-Ni-San was selectively depleted from the whole extract. This IAC column of anti-naringin antibody could be used for specifically depleting naringin from Si-Ni-San and other samples. [66]. Others application of affinity chromatography for separation of the active ingredients from TCM are shown in Table **3**.

5. BC

BC is a technology gradually developed during the 1980s. It is established based on the combination of life science and chromatographic separation techniques, and has already successfully applied to the screening of active ingredient and research on the mechanism of drug action [77]. BC has the following distinctive features: 1) it can simulate some key steps of biological activity expression of drugs *in vivo* under the physiological or pathological states. 2) The retention behavior of drugs in the biological column related directly to their activities, or their combinations with biological macromolecules, target body or cell, which have certain pharmacological or physiological significance. (3) For the research of object with complex ingredients, active ingredients can be specifically and selectively combined by BC with gel particles as the stationary phases, and simultaneously a large number of interference of impurity components can be excluded. BC has become an effective means of studying complex object [78, 79]. Table **4** shows some studies on the TCM or components separated by BC method.

5.1. Molecular-Biology Chromatography (MBC)

MBC is a new technology with the capability of integrated analysis for screening, isolation and structure elucidation of active ingredients from drugs [88-89]. MBC, using biological macromolecules as the stationary phase, is a kind of HPLC technique. The solute of retention behavior and stereo-selectivity in the stationary phase can reflect the interactions of non-fixed biological macromolecules. In fact this chromatography technique is an extension of affinity chromatography, but it is focused on studying the interaction between biological molecules, and it has been successfully applied in analysis of some active ingredients in drug [88, 90].

MBC employs active enzymes, receptors, antibodies, transport proteins and other biological macromolecules as the stationary phase to analyze and determine active ingredients and their biochemical parameters [91]. Active ingredients from nature derived drugs can be separated by the MBC with human serum albumin (HAS) as the stationary phase

Table 3. Some Applications of IAC for Separation of Active Components from TCM

Chinese herbs	Stationary phase	Active components	The results	Reference
molecular imprinting chromatography				
tea polyphenols	MIPs with EGCG as template, α-methacrylate as functional monomer, and ethylene glycol dimethacrylate as cross-link polymers.	EGCE	EGCG difficult separated by common methods can be acquired by this method.	[67]
Caragana Jubata	Used Quercetin, acrylamide, ethylene glycol dimeth extensively cross-linked polymers containing specific acrylate and MIPs with quercetin as template.	(*E*)-piceatannol and butein	The recovery rate of (*E*)-piceatannol and butein, two stronger activity than quercetin epidermal growth factor receptor inhibitor, were 80 % and 76 %.	[68]
Hippophae rhamnosidse Linn	MIPs with acrylamide as functional monomer, Quercetin as template, tetrahydrofuran as solvent, ethylene glycol 2-dimethacrylate as cross-linked polymer, and azobisiso-butyronitrile as initiator.	Quercetin and isorhamnetin	Quercetin and isorhamnetin were effectively separated by this method for the first time, and better separation for quercetin.	[69]
Polygonum cuspidatum S ieb. et Zucc.extracts	MIPs with Resveratrol as template,acrylamide as functional monomer, and azobisiso-Butyronitrile as initiator.	resveratrol; piceid	Components were obtained just two-step elution,and effectively separated.	[70]
ephedra	MIPs with (-) –ephedrine as template, methacrylate as functional monomer.	(-)-ephedrine	A good recovery, high purity and precision were proved.	[71]
tea	MIPMs (molecularly imprinted polymer microlevel) with caffeine as template.	caffeine	The results demonstrated this chromatography had the capacity of strong affinity and specific identify for caffeine, and can be used for separation caffeine in tea.	[72]
ash bark extracts	MIP-SPE with (4 mmol) acrylamide as functional monomer; ethanol as solvent; AIBN as intiator and EGDMA as template.	aesculetin	Designed a program to separate aesculetin and its structural analogues aesculin, coumarin and 7-methoxy coumarin.	[73]
IAC				
a crude extract of ginseng (*Panax ginseng*) roots	Affinity chromatography column with anti-ginsenosides Rb1 monoclonal antibody immobilized affinity gel.	Rb1	Pure extracts were acquired in the first step. The result provided evidence that a combination of two immunoassays using monoclonal antibodies against ginsenosides is a powerful means of surveying new ginsenoside resources.	[74]
Kalopanax pictus	Affinity chromatography column with anti-ginsenoside Rb1 monoclonal antibody immobilized affinity gel.	Ginsenoside Rb1	Ginsenoside Rb1 was acquired in the first step of separation, with high efficiency and easy operation.	[75]
the crude extract of *Caragana jubata*	Affinity chromatography column with piceatannol (receptor inhibitor of anti-epidermal growth factor) as hapten connected with bovine serum albumin, and preparation of the corresponding polyclonal antibodies.	anti-epidermal hapten (growth factor)	Several different structures of hapten, anti-epidermal growth factor receptor inhibitor, were effectively identified.	[76]

[92]. The results showed that, compared to common HPLC, the separation of different components from TCM in the HSA column can simultaneously eliminate a large number of interference of impurity components. Some results acquired by this way are given in Table **4**.

5.2. Cell Membrane Chromatography (CMC)

CMC, a novel bio-affinity chromatographic technique, using activity tissue or cell as stationary, originated by He [93], utilizes the specific affinity interactions between cell membrane and active ingredients to separate active ingredients from TCM. CMC can sensitively identify the bit and even trace components, including drug–receptors [94, 95]. CMC has been successfully applied in the screening of active ingredients from Chinese herbal medicine [96]. With its development, CMC will play a great role in promoting the screening and separation of active components from TCM and the clarification of action mechanisms of TCM.

Anti-atherosclerosis components from *Salvia miltiorrhiza* were screened by CMC with the stationary phase employed CD40 cell-membrane [80]. CMC allows the separation of active ingredients combined with their screening, which overcomes the past defect of starting to separate the effective parts or monomers from TCM, and then analyzing their efficacy. Table **4** shows some recent results of effective components screening by CMC method.

Table 4. Chinese Medicine or Compounds Analyzed by the BC

Chinese medicine or compound	Chromatography column	Chromatography condition	Active ingredients	The results	References
Salvia miltiorrhiza	The stable high expression of CD40 in human endothelial cells ECV-304 (300 mm ×30 mm I.D.)	flow rate 0.5 ml/min; mobile phase: 50 mmol/l PBS (pH 7.4), detection wavelength: 254 nm; column temperature: 37 °C	Tanshinone II A, Tanshinone I A and Danshensu	The model of the newly constructed cell-membrane chromatography can be used to screen drugs for atherosclerosis.	[80]
Angelica sinensis	Rabbit vascular cell membrane; Hypersil ODS (150 mm ×4.6 mm I.D., 5 μm)	mobile phase: 60 %methanol; flow rate: 1.0 ml/ min. ; column temperature:37 °C	Ligustilide dimethyl phthalate	Thus effective and sensitive screening model can be made.	[81]
Ligustum Chuanxiong	Myocardial cell membrane stationary phase (CMSP) chromatographic column (4.6 mm ×250 mm I.D.)	mobile phase: 50 mmol /l -PBS(pH 7. 4); flow rate0.3 ml /min; detection wave length278 nm; column temperature:37 °C	tetramethylpyrazine, vanillic, chrysophol	Proved the retention characteristics of tetramethylpyrazine, vanillic and chrysophol to lay the foundation for further effective separation.	[82]
Herba eplmedii	Canine vascular CMC column (150 mm×4.6 mm I.D.)	mobile phase: 50 mmol/l PBS (pH7.4); detection wavelength: 270 nm; flow rate: 1.0 ml/min.; column temperature:37 °C	Two active ingredients YYH-214 and YYH-216	Active components had obvious chromatographic peak and others had on retention characters.	[83]
Cuscuta chinensis (dodder)	Rabbit vascular CMSP (50 mm × 2 mm I.D.)	mobile phase: 50 mmol/l PBS (pH 7.4); flow rate: 0. 5 ml/min.; detection wavelength: 236 nm; column temperature: 37 °C	T2 (an active one in vasodilatation)	T2, acquired from ether extracts of *dodder,* was an active one in the vascular cell membrane and membrane receptors.	[84]
Leontice robustum	Rabbit vascular CMSP: (50 mm × 2 mm I.D.)	mobile phase: 50mmol/lPBS (pH 7. 4); flow rate: 0.5 ml/min.; detection wavelength: 256 nm; column temperature: 37 °C	HMQ-44	HMQ-44 separated from *Leontice robustum* and proved was an active one in vasodilatation.	[85]
Cladonia alpestris	Vascular and myocardial CMC column (50 mm ×2 mm I.D.)	mobile phase: PBS (pH 7.4); flow rate: 0. 5 ml/min.; detection wavelength: 236 nm; column temperature: 37 °C	TBH2-2,TBH2-6 and TBHG8	TBH2-2 and TBH2-6 from the part of Ether extracts were active one in the vascular cell membrane and membrane receptors; the active ingredient (TBHG8) from petroleum ether extracts of *Cladonia alpestris* was an active one in myocardial cell membrane and membrane receptors.	[86]
Carthamus tinctorius L. (safflower)	CMC column: (50 mm×2 mm I.D.)	mobile phase: 50 mmol/l PBS(pH 7. 4); flow rate: 0. 5 m l/min.; detection wavelength: 210 nm; column temperature: 37 °C	HH- S, HH- S2	It was found that the HH-S was an effective part and the component (HH-S2) separated from the part was an active one in vasodilatation.	[87]

The amount of active ingredients from TCM isolated by CMC, namely the amount of active ingredients specifically retains in column of CMC, is less than that of other methods. Therefore, there are many aspects need to be improved and enhanced in the process of screening the active ingredients by CMC. It could be considered to expand the scope of op-

tional cell membranes to prepare the retinal cells, nerve cells and tumor cells, etc. as the membrane stationary phase. HPLC- diode array detection (HPLC-DAD) detector and a higher sensitive HPLC-MS can be used to analyze the active ingredients of TCM and to synchronously complete the isolation and structural identification of active ingredients, hence to increase the screening efficiency.

6. CONCLUSIONS AND PROSPECTS

Different chromatographic techniques have their own different advantages in separating the active ingredients from TCM. 1) Liquid chromatography, including UPLC, RRLC, and HSCCC has high analysis speed, sensitivity, and efficiency; 2) UPLC plays a significant role in the separation, analysis and purification of some thermo-sensitive, volatile and polarity components; 3) The specificity of AC can be used to analyze and effectively separate the targeted compounds from mixture; 4) While screening active ingredients, BC can excluded a large number of interference caused by inactive impurities. In dealing with large quantities of complex components of TCM, chromatographic technique has played a significant role in the TCM preparation, identification, active component analysis, and the quality control.

But at present the chromatographic techniques mentioned above exist some deficiencies: 1) LC mainly depends on reducing filler particle size to achieve the goal of rapid analysis and separation, but the column pressure of 1.7 μm particles has already reached 103.4 Mpa, further reducing the particle size to improve efficiency is nearly impossible. The investigations on the choice of mobile phase and the system suitability should be strengthened; 2) UPLC can not be used independently for the detecting and separating active ingredients, it is usually employed as an auxiliary method in the field of chromatography. It has strict requirements for the composition of buffer solution in the mobile phase, column pressure, and column temperature, and these parameters can not be adjusted easily; 3) types of stationary phase vectors of AC is still limited, thus limits the separation of a number of active ingredients. A variety of antibodies materials should be developed as affinity chromatography column. 4) The lifetime of Bio-column is usually short, about 1-15 d; stationary phase has not yet achieved commercialization, some bio-columns have to be prepared in laboratory with certain conditions. Understanding about biological characteristics of chromatography of most chromatography methods do not go far enough.

In future, much attention will be paid on the combination of chromatography with MS, tandem MS and other technologies, thus chromatography method will be progressively developed and improved in separation of the active ingredients from TCM. Chromatography techniques will not only be widely applied in the laboratory, but also has great potential in the field of large-scale preparative chromatography. In addition, more efforts should be put on reasonable combination of various chromatographic separation methods, and this is an important subject and direction of future research.

ABBREVIATIONS

AC = affinity chromatography

API = atmospheric pressure chemical ionization

BC = bio-chromatography

CMC = cell-membrane chromatography

DAD = diode array detection

GC = gas chromatography

HPAC = high-performance affinity chromatography

HPLC = high-performance liquid chromatography

HAS = human serum albumin

HSCCC = high-speed counter-current chromatography

IAC = immunoaffinity chromatography

LC = liquid chromatography

MBC = molecular-biology chromatography

MS = mass spectrometry

Prep-SFC = preparative-supercritical fluid chromatography

RRLC = rapid resolution liquid chromatography

SF = supercritical fluid

SFC = supercritical fluid chromatography

TCM = traditional Chinese medicine

TOF = time-of-flight

UPLC = ultra-performance liquid chromatography

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