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Review

Advances in Application of High-Speed Countercurrent Chromatography in Separation and Purification of Flavonoids

DI Duo-Long[1,*], ZHENG Yuan-Yuan[1,2], CHEN Xiao-Fen[1,3], HUANG Xin-Yi[1], FENG Shi-Lan[2,*]

[1] Key Laboratory of Chemistry of Northwestern Plant Resources & Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China
[2] Lanzhou University Pharmaceutical College, Lanzhou 730000, China
[3] Graduate University of the Chinese Academy of Sciences, Beijing 100049, China

Abstract: High-speed countercurrent chromatography (HSCCC) is a novel, efficient, and productive chromatographic technique based on continuous liquid–liquid partition, which has the unique feature of eliminating the irreversible adsorption using liquid support, and is widely used in the fields of traditional Chinese medicine, biochemistry, food, natural product chemistry, environment analysis, and so on. In this study, the application of HSCCC in the separation and purification of known and unknown flavonoids is reviewed. Furthermore, this study introduces the developments in HSCCC techniques and their application in the separation and purification of flavonoids and other compounds.

Key Words: High-speed countercurrent chromatography; Flavonoids; Review

1 Research on application of HSCCC in separation and purification of flavonoids from natural products

Flavonoids are significant chemical components of plants, which are important active components in many traditional Chinese medicines and in phytomedicine. The basic mother nucleus of flavonoids is 2-phenyl-chromone, flavonoids including flavone, isoflavone, flavanone, anthocyanidins, flavanol, aurone that belongds to the isomers of flavonoids, and their various derivatives. Flavonoids such as rutin, quercetin, and quercitrin possess significant physiological and pharmacological activity, which can strengthen the contraction of heart or cardiac muscles and reduce the fluctuations of heart. Baicalin and luteolin have antibacterial and antiphlogistic effect; naringenin has estrogen activities. Licochalcone shows anti-HIV activity[1]. In addition, flavonoids can also be used as a functional food additive and a natural antioxidant. Because of their effectiveness outlined earlier, the separation and purification of flavonoids from natural products is of great significance.

In plants, flavonoids are of a variety of kinds, are present in a low amount, have complex chemical composition, and exhibit a variety of biological activities. Therefore, it is extremely important and challenging to separate and purify flavonoids from plants. Generally, conventional methods, such as liquid-liquid extraction, column chromatography (silica gel column, polyamide column, Sephadex column), macroporous resin, preparative/semipreparative reversed-phase liquid chromatography, are used for isolation and purification of flavonoids from plants. However, these methods are tedious, consume time and solvent, and require multiple chromatographic steps; therefore, it is difficult to realize industrialization[2,3] of these methods. High-speed counter-current chromatography (HSCCC), which was based on liquid-liquid distribution chromatography, was developed as a modern chromatographic separation and preparation technology; it was developed in the beginning of 1980s by Dr.

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* Corresponding author. Email: didl@licp.cas.cn
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Yiochiro Ito[4], the National Institutes of Health (NIH), United States. Because of its sustained high efficiency, high recovery, and ability for preparation of a large amount of compounds, HSCCC can be directly applied to crude extract, to separate the compounds that rely only on their different solubility properties, and to avoid the shortages such as sample loss caused by irreversible adsorption and analyte degeneration caused by surface chemistry. Therefore, HSCCC was pointed out for wide application in the fields of purification and separation, especially extraction and separation of active components from plants; now it has become a novel, worldwide separation and purification technique.

In recent years, HSCCC has been broadly applied for the separation and purification of flavonoids from plants due to its unique advantages. Svtherland *et al*[5] reviewed about 198 literatures on the separation and purification of effective ingredients from plants using HSCCC: a total of 363 compounds were isolated from 108 plants, the number of flavonoids separated from those plants accounts for about 25% of the 363 compounds, and 89 articles reported on the separation and purification of flavonoids. Thus, it can be seen that HSCCC has a significant role in the separation and purification of flavonoids from plants. Table 1 partly summarizes the research reports on the separation and purification of flavonoids from plants using HSCCC.

2 Application of different HSCCC techniques in separation and purification of effective ingredients from natural products

2.1 Cross-axis countercurrent chromatography[27]

Compared with traditional HSCCC, cross-axis countercurrent chromatography (Cross-axis CCC) showed that the spin axis

Table 1 Application of HSCCC in separation and purification for flavonoids in recent years

Plants	Compounds	Solvent system (*V/V*)	Flow rate (mL min^{-1})	Revolution speed (rpm)	Ref.
Flos Gossypii	Tiliroside, quercetin, kaempferol	*n*-Hexane-ethyl acetate-methanol-water (1:2:0.8:0.9)	1.0/4.0	800	[6]
Hypericum perforatum L.	Quercitrin, quercetin, hyperoside, isoquercitrin, miquelianin, epigallocatechin, avicularin	Ethyl acetate-methanol-water (10:1:10)	2.0	800	[7]
A. membranaceus Bge. var. *mongholicus* (Bge.) Hsiao	Calycosin-7-*O*-b-*D*-glycoside, formononetin-7-*O*-b-*D*-glycoside	Ethyl acetate-ethanol-water (5:1:5)	1.0	1800	[8]
Smilacina japonica A. Gray	Luteolin, quercetin	Chloroform-methanol-water (4:3:2)	2.0	800	[9]
Acer truncatum Bunge	Quercetin-3-*O*-L-rhamnoside	Ethyl acetate-ethanol-water (5:1:5)	2.0	800	[10]
Artemisia annua L.	Casticin	*n*-Hexane-ethyl acetate-methanol-water (7:10:7:10)	2.0	800	[11]
leaves of *Byrsonima crassa* Niedenzu (IK)	Quercetin-3-*O*-*α*-*L*-arabinoside, quercetin-3-*O*-*β*-*D*-galactoside, biflavonoid amentoflavone	Ethyl acetate-*n*-propanol-water (140:8:80)	3.0	800	[12]
Taraxacum mongolicum	Alquds, hesperidin	*n*-Hexane-*n*-butanol-water (1:1:2)	1.5	800	[13]
Sarcandra glabra	Isofraxidin	*n*-Hexane-ethyl acetate-Methanol-water (1:2:1:2)	2.0	800	[14]
Apple tree bark	Phloretin	*n*-Hexane-ethyl acetate-ethanol-water (2:2:1:2)	2.0	800	[15]
Honeybush (*Cyclopia subternata*)	Isomangiferin	MTBE-*n*-butanol-MeCN-water (3:1:1:5)	3.0	850	[16]
Patrinia villosa Juss	Isovitexin, isoorientin	Ethyl acetate-*n*-butanol-water (2:1:3)	2.4	800	[17]
Oroxylum indicum	Fbaicalein, chrysin	Petroleum ether-ethyl acetate-methanol-water (5:5:5:5)	2.0	850	[18]
Pericarpium Citri Reticulatae	Hesperidin, tangeretin, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone	Petroleum ether-ethyl acetate-methanol-water (2:4:3:3)	1.7	850	[19]
Fructus aurantii	Naringin	Ethyl acetate -*n*-butanol-water (2:0.8:3.2)	1.5	850	[20]
Sea Buckthorn Juice Concentrate*(Hippophaë rhamnoides L. ssp. rhamnoides)*	Isorhamnetin 3-*O*-b-*D*-glucoside, isorhamnetin 3-*O*-b-rutinoside, quercetin 3-*O*-b-D-glucoside, protocatechuic acid	*n*-Hexane-*n*-butanol-water (1:1:2)	3.0	800	[21]
Selaginella moellendorffii	Amentoflavone, hinokiflavone, podocarpusflavone A, ginkgetin	*n*-Hexane-ethyl acetate-methanol-water (8:8:9:7)	1.2	800	[22]
Helichrysum arenarium (L.) Moench	Naringenin-7-*O*-b-Dglycoside, isoquercitrin, astragalin	Ethyl acetate-water (1:1)	1.5	800	[23]
Rhizoma Anemarrhenae	Mangiferin, neomangiferin	*n*-Butanol-acetic acid (1:1)	2.0	700	[24]
Patrinia villosa Juss.	Orotinin, orotinin-5-methyl ether, licoagrochalcone B	*n*-Hexane-ethyl acetate-methanol-water (5:6:6:6)	0–180 min, 1.0; 180 min, 2.0	900	[25]
Soybeans	Daidzin, genistin, 6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin	*n*-Hexane-ethyl acetate-*n*-butanol-methanol-acetic acid-water (1:2:1:5:1)	2.0	700	[26]

of spiral support parts was mutually perpendicular to the public shaft of the instrument and that three-dimensional asymmetric centrifugal force fields developed when planetary motion occurred. This special structure made it very beneficial to retain the stationary phase of the hydrophilic solvent system, which was applied to the separation and preparation of biomacromolecules. Cross-axis CCC has advantages in retaining the stationary phase and the separation efficiency compared with conventional HSCCC, especially in the fields using the hydrophilic solvent system to separate strong-polarity compounds and an aqueous two-phase solvent system to separate hydrosoluble biomacromolecules. Cross-axis CCC has been successfully applied in the isolation of macromolecular compounds, such as various proteins, bio-enzymes, and polysaccharides. Wei *et al*[28] used Cross-axis CCC to separate three glycoproteins from the fermentation media of *Morchella esculenta* (L.): the solvent system was composed of 12.5% (*w/w*) polyethylene glycol 8000 and 25% (*w/w*) potassium phosphate in distilled water (pH = 9) under the conditions of 1.0 mL min^{-1} flow rate and 500 rpm rotation speed. The overall results of the studies indicated that the Cross-axis CCC provided a new method for the preparative separation of natural, biological macromolecules from plants and increased sample injection rate with the prerequisite of decreasing the sample loss and not destroying the active ingredients. At present, literatures document about the application of Cross-axis CCC to the separation of proteins, enzymes, and polysaccharides, but the studies about the application of Cross-axis CCC on the separation of flavonoids are exceedingly rare. The successful separation of three glycoprotein components from *Morchella esculenta* (L.) showed that Cross-axis CCC has extensive prospect of application in the separation of natural biological macromolecules and other active ingredients from plants.

2.2 Dual countercurrent chromatography

Dual countercurrent chromatography (DuCCC) is an improved version of the traditional HSCCC. In DuCCC, both the liquid phases are mobile phases. DuCCC comprises a moving bed or countercurrent flow with the light and heavy liquid phases moving through the coil in opposite directions in the true sense. DuCCC has the following advantages: (1) It achieves two kinds of different separation modes: forward and reverse modes; (2) Two-phase solvent used in the spiral pipe is always clean; there are no problems of loading quantities and stationary phase saturation, and DuCCC makes successive sample injection possible; and (3) DuCCC is suitable for the preparative separation of the complex mixture system and simultaneous efficient separation of polar and nonpolar composition. In addition, DuCCC has a very prominent feature that it can use not only liquid-liquid as two-phase mobile phase, but also gas-liquid as two-phase mobile phase. This separation technique is named foam countercurrent chromatography (FCCC), which can be used to separate biological samples with high water solubility[29]. Ito *et al*[30] used a two-phase solvent system of *n*-hexane-acetonitrile to separate *N*-methylcarbamate pesticides from vegetable oil and citrus fruit. Under optimum conditions (the flow rate was 2.0 $mL \cdot min^{-1}$, the coiled column rotated at 420 rpm), the goals of simultaneous isolation and purification of carbaryl, fenobucarb and methomyl were achieved. This unique feature of DuCCC helps in establishing the rapid sample preparation method by repetitive sample injection with high reproducibility without risk of contamination from the components retained in the column. It is believed that the present method has a great potential in analyzing both hydrophilic and hydrophobic compounds in food, environment, and biological matrix. Through screening and improving two-phase mobile phase and improving DuCCC instruments, the application fields of DuCCC in the separation and purification of effective components from natural products would be widened.

2.3 pH zone refining countercurrent chromatography[31]

pH zone refining countercurrent chromatography (pH zone refining CCC), which was developed as a special separation technique in the 1990s, had greatly widened the application fields of HSCCC. According to the different dissociation constants (pK_a) and hydrophobicity of the compounds, the chemical compounds are separated using pH zone refining CCC. pH zone refining CCC is mainly used for the separation of alkaloids, organic acids, and amino acid and its derivatives. Compared with the traditional HSCCC, pH zone refining CCC has the following advantages: (1) increased sample loading capacity, (2) high concentration of fractions, (3) and higher separation efficiency. The UV absorption spectrum of compounds is changed through adjusting the pH values so that the compounds without UV absorption can also be on-line detected. Still, pH zone refining CCC realized the easy detection of minimum components by concentrating them during the separation procedure. Wang *et al*[32] used MtBE-CH_3CN-water (4:1:5, *V/V*) as the two-phase solvent system. The organic stationary phase was made acidic with 10 mM of trifluoroacetic acid, and the lower mobile aqueous phase was alkalized by addition of aqueous ammonia to obtain 10 mM of ammonia solution. Double separations were performed to obtain cichoric acid with anti-HIV activity from *Echinacea purpurea* using pH zone refining CCC (the purity of the separated product was 87.8% after the first operation step, and the purity was increased to 95.6% after the second step). The successful isolation of cichoric acid emphasized that pH zone refining CCC can be used to separate organic acid from natural products.

Owing to the fact that the molecules of flavonoids have the

phenol hydroxyl group and that the 1-oxygen atom in γ- pyran ring has an unshared electron pair, flavonoids are weakly acidic. pH zone refining CCC is restricted to separate the acids and alkali compounds with obvious difference in dissociation constants[33]. Consequently, the application of pH zone refining CCC in the separation and purification of flavonoids is blocked. Some literatures reported that by adding some suitable acidic/alkaline extractant or mixed ionic solvent to the flavonoids so as to form complex compounds which could be separated with pH zone refining CCC. Thus, the distribution coefficient of compounds was changed so that the purpose of separation and purification was achieved. The authors of this study have not yet come across reports on the application of pH zone refining CCC in the separation and purification of flavonoids from plants, but it is undeniable that its application will have great potential and wide prospect.

2.4 Coupling techniques of HSCCC

In the procedure of separating natural compounds using HSCCC, a UV detector is commonly used as the data acquisition device, which restricts the detection of chemical composition with weak or no UV absorption. Evaporative light scattering detector (ELSD), as a universal detector, can detect any samples whose volatility is lower than that of the mobile phase, and it does not need chromophoric groups in compounds. Therefore, the application of ELSD in HSCCC becomes more and more wide. With the development of research, the combination of HSCCC technique with mass spectrometry (MS), Fourier transform infrared spectroscopy (FTIR), and other new kinds of detectors provide new separation and analysis methods, and thus, powerful techniques support the preparation of standards, establishment of fingerprints and determination of chemical composition of natural medicine, and development of new drugs. In addition, HSCCC has been successfully combined with sample pretreatment techniques.

Chen *et al*[34] reported that baicalein, baicalein-7-Oglucoside, and chrysin were separated successfully from the ethyl acetate extract of the seeds of Oroxylum indicum utilized an analytical HSCCC interfaced with APCI- and ESI-MS mode, which used a solvent system composed of hexane-ethyl acetate-methanol-0.2% formic acid (1:1.2:1:1, *V/V*). HSCCC/MS, which combines the advantage of HSCCC with the separating multiplicity and lower detection limit and the advantage of MS with high sensitivity, can quickly provide useful structure information for the identification of the compounds. This technique supports the separation and analysis of effective components and fast determination other ingredients from natural products, drugs, protein and other biochemical substance. Gutzeit *et al*[35] used preparative HSCCC/ESI-MS- MS for a fast screening and fractionation of polyphenols, including flavonol glycosides and protocatechuic acid from sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*, Elaeagnaceae). The two-phase solvent system was composed of *n*-hexane-*n*-butanol-water (1:1:2, *V/V*), and there was a T-splitter unit between the fraction collector and ESI-MS-MS detector. When the mobile phase effused from HSCCC, the split ratio was adjusted according to the different inlet sample quantity. T-splitter unit was established to avoid the problems of overload and contaminate, caused by excessive amounts of samples was injected into MS. The experiment results demonstrated that HSCCC/ESI-MS- MS could be used as a versatile on-line detecting technique for separation and purification and that it offers the MS information of effective components in natural products.

In recent years, HSCCC became more and more important in the separation and purification of natural active ingredients; the technique of HSCCC interfered with various detectors and analytical devices became gradually perfect. Combined with the needs of practical work, HSCCC and its coupling techniques will be further developed and will have more space and broad prospects in various applications.

3 Application of HSCCC in separation and purification of flavonoids

3.1 Application of HSCCC in separation and purification of known flavonoids from natural products

With the development of countercurrent chromatography, HSCCC is exploited not only as an extraction technique but also as a concentration method to trace compounds from a large volume of solvent. One of the main areas of application of countercurrent chromatography is the separation and purification of bioactive ingredients from natural products in quantities sufficient for the later study of their biological, pharmacological, and clinical effects both in vitro and in vivo[36]. Flavonoids are a kind of compounds with many bioactivities, and there are numerous reports on the usage of HSCCC to separate and prepare the known flavonoids[5].

Xiao *et al*[37] used HSCCC to separate and purify five flavonoid glucosides from *Radix Astragali* through two steps. In step 1, the solvent system of *n*-hexane-ethyl acetate-*n*-butanol-methanol-0.5% TFA (1:2:1:1:5, *V/V*) was used to separate compound 1, and *n*-hexane-ethyl acetate-*n*-butanol-methanol-0.5% TFA (2:3:1:1:5, *V/V*) system was applied to separate compound 5 from other compounds, with 800 rpm rotation speed. Chloroform-methanol-water (4:3:2, *V/V*) system was used for the separation of compounds 2–4 in the next step with 600 rpm rotation speed, and the flow rate in the two steps was 2 mL min^{-1}. The separated five compounds were calycosin-7-*O*-*β*-d-glucoside, ononin, (6aR,11aR)-9,10-dimethoxypterocarpan-3-*O*-*β*-d-glucoside, (3R)-2′-hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-*β*-d-glucoside, and calycosin-7-

O-*β*-d-glucoside-6″-O-acetate, and the purity was 97.92%, 87.2%, 99.5%, 96.3% and 99.35%, respectively. HSCCC overcame the shortcomings of traditional silica column chromatography, such as tediousness, consumption of time and solvent, and requirement of multiple chromatographic steps. This study demonstrated that HSCCC has a great potential to the preparative isolation of flavonoid glucosides from *Radix Astragali* and to discover monomeric compounds from traditional medicines.

There are reports and literatures on the separation and purification of known flavonoids from natural products, and they are partly reviewed in Section 1 and Table 1 as it is unnecessary to go to complete details.

3.2 Application of HSCCC in discovering new flavonoids from natural products

Since the countercurrent chromatography was developed, the application of HSCCC to separate and purify new compounds from plants had been reported. The relevant reports on new flavonoids also accounted for a large percentage. The following content summarizes some reports on the separation and purification of new flavonoids by HSCCC.

Zhao *et al*[38] reported that 52 mg of one new flavonoid glycosides with purity of 98.0% was obtained from 2.0 g of ethyl acetate extract of the flowers of *Carthamus tinctorium* (Honghua). The optimized conditions were as follows: two-phase solvent system was composed of ethyl acetate-methanol-water (5:1:5, *V/V*), and the flow rate and rotation speed were 2.0 mL min^{-1} and 800 rpm, respectively. The structure of the target compound was elucidated by means of spectroscopic methods, such as IR, MS, 1D, and 2D NMR techniques, the result showed that it was (2S)-4′,5,6,7-tetra-hydroxy-flavavone-6-O-*β*-d-glucopyranoside. The research showed that HSCCC avoided sample loss caused by the irreversible adsorption in conventional silica gel chromatography in the process of separating new minor components. Peng *et al*[39] successfully separated three flavonoids including a novel flavanone from *Patrinia villosa Juss* using two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (10:11:11:8, *V/V*) at the flow rate and rotation speed of 1.8 mL min^{-1} and 800 rpm, respectively. The new compound obtained was 5,7,2′,6′-tetrahydroxy-6,8-di(γ,γ-dimethylallyl) flavanone with purity of 99.2% and its structure was identified through UV, IR, MS, 1H NMR, 13C NMR and 2D NMR. In 2006, Peng and his co-workers[40] used the solvent system of *n*-hexane-ethyl acetate-methanol-water (10:13:13:10, *V/V*), the rotation speed was set at 900 rpm, six flavonoids including two known and four novel compounds were successfully and simultaneously purified from the leaves of *Patrinia villosa Juss* by adjusting the current gradient (increasing the flow rate of the mobile phase from 1.0 mL min^{-1} to 2.0 mL min^{-1} after 110 min to exude the later eluters). Various spectrum methods such as UV, IR, HR-ESI-MS, 1D and 2D NMR were employed for identifying the structures, and the four novel compounds obtained were (2*S*)-5,7,2′,6′-tetrahydroxy-6-lavandulylated flavanone, (2*S*)-5,7,2′,6′-tetrahydroxy-4′-lavandulylated flavanone, (2*S*)-5,2′,6′-trihydroxy-2″,2″-dimethyl-pyrano [5″,6″:6,7] flavanone, and (2*S*,3″*S*)-5,2′,6′-trihydroxy-3″-γ,γ-dimethylallyl-2″,2″-dimethyl-3″,4″-dihydropyran [5″,6″:6,7] flavanone with purity of 98.8%, 99.3%, 98.8%, and 98.6%, respectively. Peng et al. separated five new flavonoids from *Patrinia villosa Juss* with high purity through HSCCC. This method showed the advantages of HSCCC: simple operation, time saving and low cost, high preparative capacity, provision of all information of samples from plants, and so on. HSCCC can also provide sufficient samples to evaluate the anticancer activities in vitro in latter experiment. Peng *et al*[41] used petroleum ether-ethyl acetate-ethanol-water (1:1.2:1.2:1, *V/V*) as the solvent system at a flow rate of 2.0 mL min^{-1} and the apparatus was controlled at 800 rpm. Under the optimized conditions, two new prenylated dihydroflavonoids were isolated from 400 mg of *Dolichos tenuicaulis (Baker) Craib* through HSCCC. The two novel dihydroflavonoids were (2S)-5,2′,6′-trihydroxy-8-prenyl-6,7-(3-prenyl-2,2-dimethyl-1-keone-cyclohexadiene) flavanone, (2S)-5,2′,6′-trihydroxy-8-prenyl-6,7-(3-prenyl-2,2-dimethylpyrano)-3′,4′-(2,2-dimethyl-1-keone-cyclohexadiene) flavanone, and they were elucidated through spectroscopic methods such as UV, IR, ESI-MS, 1D NMR, and 2D NMR. Yuan *et al*[42] optimized the experiment conditions on analytical HSCCC as follows: the solvent system was composed of chloroform-methanol-water (9.5:10:5, *V/V*), the flow rate was 1.5 mL min^{-1}, and the rotation speed was 1800 rpm. The optimized conditions were amplified to preparative HSCCC, and a new chrysin-diglucoside (21.7 mg, 98.8% in purity) was isolated from 911.6 mg ethyl acetate extract of *Oroxylum indicum*. The structure of chrysin-diglucoside was identified through HPLC, ESI, MS, and NMR. The amplification of this experiment kept the original resolution and separation time. Under the premise, the handling capacity of preparative HSCCC increased 53 times compared with analytical HSCCC. This method provided efficient separation and purification methods for natural products.

3.3 Application of HSCCC in screening active ingredients from natural products

HSCCC is a continuous and highly efficient liquid–liquid partition chromatography, and the separation process could be completed within few hours. The mild operation condition of it avoided inactivity and decomposition of active ingredients, and there is no irreversible surface adsorption effect because of the inexistence of solid support. Meanwhile, there is no

change of raw ingredient in extracts.

The authors of this study used offline hyphenated techniques of HSCCC-HPLC to screen and separate active material from leaves of *Olea europaea* L.[43]. The method of DPPH-HPLC-DAD was used to screen the major radical scavengers by contrasting the change of the HPLC-DAD chromatograms of the blank sample and the spiking sample, which confirmed that compounds 1, 2 and 3 possessed antioxidant activity. Three compounds were obtained through HSCCC under the optimum conditions, and chemical structure information of active monomers was identified through ^{1}H-NMR and ^{13}C-NMR. In the aforementioned study, the combination of DPPH-HPLC with HSCCC was performed, and three major constituents with antioxidant activity were screened, isolated and identified by DPPH-HPLC and HSCCC from herbal medicines. Yuan *et al*[44] separated three flavonoids (apigenin, camellianins A and B) from Adinandra nitida leaves through HSCCC. The cytotoxicity of three flavonoids against human epidermal carcinoma cancer A431 cells was evaluated by using the MTT method, and the results explained and pointed out that the plant resources possessed antitumor activity and main effective ingredients.

4 Prospect

Although HSCCC is a kind of new chromatographic technique for separation and purification of flavonoids from natural products, it has a development history of only 20 years. As a liquid-liquid partition chromatographic technique with unique superiority, the development of HSCCC is rocketing. This technique became more comprehensive and consummate. Therefore, HSCCC has wide applications in many fields because of its distinctive advantages in the separation and purification of effective components from plants. This study reviewed the latest development and application of HSCCC in the separation and purification process of flavonoids from plants. There are no limitations of the application of countercurrent chromatography to separate and purify flavonoids, and it can be used in separating all kinds of flavonoids. The main reasons are that HSCCC has plenty of two-phase solvent systems, the extraction method is flexible and diverse, and the mobile phase can use not only a single mobile phase but also a different mobile phase for each run. Two-phase solvents flowing simultaneously in opposite direction can be chosen; therefore, continuous procedure of countercurrent chromatography was realized. In addition, HSCCC also realizes multiple forms of the gradient elution process; thus it can be used not only to remove impurities from crude extract of plants but also to purify the final product. Moreover, some pure compounds can even be obtained through one step from crude extract without sample pretreatment. All these advantages are concretely embodied in the above application examples. As a new separation and purification technique, HSCCC attracts more and more researchers to concern and join in this field. Thus, HSCCC will get more wildly application and development.

Although countercurrent chromatography has many merits, other chromatographic separation methods cannot supersede. However, with the development of countercurrent chromatography, there are many factors restricting the wider application of countercurrent chromatography. Firstly, the choice of solvent systems has no mature and systematic theory guidance. Although some researchers have established several empirical methods for screening the solvent system[45], these methods are summarized on empirical regularity; determining the rules on how to choose an excellent solvent system with good separation efficiency still needs considerable experience. For the choice of a better solvent system, many experiments usually need to be carried out. Moreover, the current HSCCC realizes the preparation and separation of few hundreds milligrams or several decagrams of compounds, but there is still a long way for the preparation of several hectograms or kilograms of samples. In addition, in consideration of the applications of HSCCC in pilot scale and industrialized production, the separation mechanism of countercurrent chromatography required in-depth theoretical investigation and some key technical problems that appear in the industrial amplification of the existing instruments still need to be solved. Currently, part of the international scientific and technical personnel has been devoted to this subject, it is believed that HSCCC will develop into a more mature, manfacturable, and highly efficient separation technique in the near future.

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