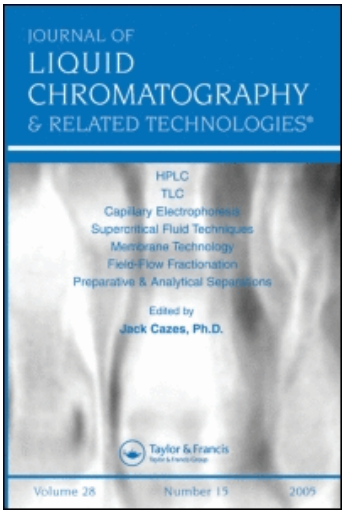

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Recent Progress in Countercurrent Chromatography

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Recent Progress in Countercurrent Chromatography

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Abstract: Countercurrent chromatography is a powerful separation technique, which is used as an alternative or complementary technique to other chromatographic methods due to its high efficiency and preparative capacity. This technique does not require a solid stationary phase and relies simply on the partition of a sample between the two phases of an immiscible solvent system. Thus, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. With these advantages, CCC is gaining popularity as an important separation method. This review summarizes the basic principles and gives the most recent CCC applications in various areas, including the isolation and purification of natural products, development of different elution modes and multidimensional methods, progress in pH-zone-refining CCC techniques, bioseparation with aqueous polymer two-phase systems, enantioseparation with various efficient chiral selectors, and on-line monitoring of the eluate.

Keywords: Countercurrent chromatography, Elution mode, Multidimensional, pH-Zone-refining, Bioseparation, Enantioseparation, On-line monitoring

INTRODUCTION

Late in the 1960s, Ito developed a new separation technique called countercurrent chromatography (CCC).[1,2] This technique is an all-liquid method without solid phases, which relies on the partition of a sample between two immiscible solvents to achieve separation. The relative proportion of solute passing into each of the two phases is determined by the respective partition coefficients.

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Therefore, CCC benefits from a great advantage when compared with the traditional liquid-solid separation methods: (1) it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination; (2) it is a very economical method (the instrument is relatively cheaper than HPLC, no expensive columns are required, low solvent consumption, and only common solvents are consumed).

The development of efficient CCC instruments has required over 30 years of steady effort by a number of scientists and engineers: the first CCC model was called toroidal coil CCC,[2] which had a rotary seal and the effluent was introduced from the rotating syringe. Although, this analytical model yielded thousands of theoretical plates, it always required an overnight separation time. The second model, droplet CCC,[3] could produce a preparative separation at nearly 1000 theoretical plates, but needed several days to accomplish a single run. Thus, the performance of these early models produced a long standing false image that CCC is a time consuming technique. In the intervening years, with the development of high-speed CCC (HSCCC),[4–6] the separation efficiency of this method has been dramatically improved in terms of resolution, separation time, and sample loading capacity. HSCCC, which is one form of CCC, is now accepted as an efficient preparative technique, and widely used for separation and purification of various natural and synthetic products.

Although, the efficiency (as represented by the number of theoretical plates) cannot match that of HPLC, the high selectivity and high retention of the stationary phase make the CCC method a valid alternative or complementary technique to HPLC, and to be a powerful preparative chromatographic tool.[7] During the past 30 years, a number of publications including monographs,[8–12] encyclopedia,[13,15] and review articles,[16,17] in addition to a great number of research articles on CCC in chromatographic journals have been published. Today, CCC is gaining popularity as an important separation method. This review will give a brief overview of CCC and survey the recently published CCC applications in various areas. Each area will provide an overview of the separation principles and give some important applications. The references given in the paper represent only a segment of the current literature available. Additional references can be found in the publications cited.

CCC FUNDAMENTALS

Type-J Multilayer Coil Planet Centrifuge

Today, HSCCC is the most advanced and widely used form of the CCC system using a multilayer coil separation column, which undergoes a type-J synchronous planetary motion. The mechanism of this motion was described in many research articles and reviews.[4–6,16] Thus, this paper only gives a brief

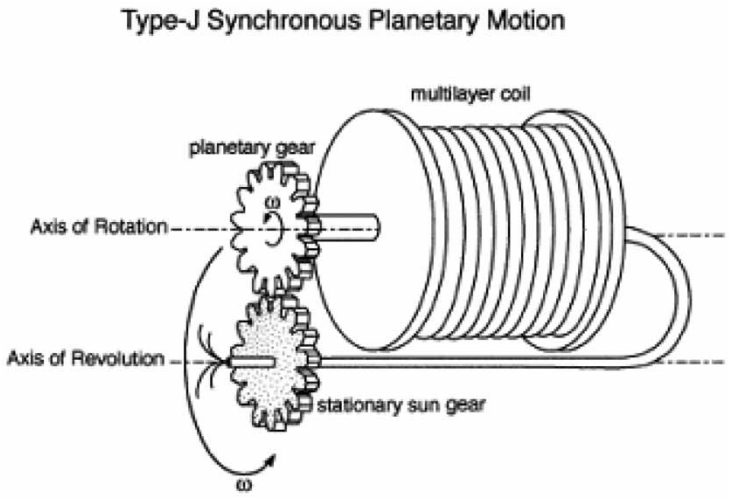

Figure 1. Type-J planetary motion of a multilayer coil separation column. The column holder rotates about its own axis and revolves around the centrifuge axis at the same angular velocity (ω) in the same direction. This planetary motion prevents twisting the bundle of flow tubes allowing continuous elution through a rotating column without risk of leakage and contamination. (Reprinted from ref. 16 with permission).

overview of the type-J synchronous planetary motion. Figure 1 schematically illustrates the type-J synchronous planetary motion of a multilayer coil separation column. The planetary motion is produced by engaging a planetary gear mounted on the column holder axis to an identical stationary sun gear rigidly fixed to the centrifuge framework. This 1 : 1 gear coupling produces a particular type of planetary motion of the column holder, i.e., the holder rotates about its own axis while revolving around the centrifuge axis at the same angular velocity (synchronous) in the same direction. This planetary motion provides two major functions for performing CCC separation: a rotary-seal-free elution system, so that the mobile phase is continuously eluted through the rotating separation column. The second and more important function is that it produces a unique hydrodynamic motion of two solvent phases within the rotating multilayer coiled column, mainly due to the Archimedean screw effect. When two immiscible solvent phases are introduced in an end-closed coiled column, the rotation separates the two phases completely along the length of the tube where the lighter phase occupies one end called the head and the heavier phase, the other end, called the tail. (Here, the head and tail relationship is defined according to the Archimedean screw effect: all objects with different densities, either lighter or heavier than the suspending medium, present in the rotating coil are driven toward the head of the coil.) When the coil is first entirely filled with the upper phase and the lower phase is pumped through the head end or alternatively, the coil is entirely filled

with the lower phase and the upper phase is pumped through the tail end, the system can maintain a high retention level of the stationary phase against a high flow rate of the mobile phase. Thus, this CCC scheme is capable of yielding efficient separations in a shorter elution time.

Choice of the Solvent System

In contrast to conventional liquid chromatography, the CCC technique uses a two-phase solvent system made up of a pair of mutually immiscible solvents, one used as the stationary phase and the other as the mobile phase. The use of two-phase solvent systems allows one to choose solvents from an enormous number of possible combinations. Therefore, the selection of a suitable two-phase solvent system is the key element in the CCC method development. However, the main difficulty also arises from the choice of the solvent in an enormous number of possibilities available to the analyst.

Generally speaking, the previous articles on CCC involving separation of similar compounds should be consulted first. When the search for a solvent system is unsuccessful, one must resort to a tedious trial to find a suitable two-phase solvent system. The selected solvent system should satisfy the following requirements: (1) short settling time ($<$30 s); (2) no decomposition or denaturation of the sample; (3) sufficient sample solubility; (4) suitable partition coefficient (K) values (usually between 0.5 and 2); (5) satisfactory retention of the stationary phase.[8–10]

A practical and effective strategy for systematically searching the solvent systems in CCC developed by Ito et al.[16,18] is recommended: two sets of two-phase solvent systems are arranged from top to bottom in decreasing order of hydrophobicity in the organic phase. The search is according to the polarities and the K values of the analyte. For example, when the polarity of the target compounds is unknown, the search may start with the two-phase solvent system composed of hexane-ethyl acetate-methanol-water at a volume ratio of 3 : 5 : 3 : 5, which has a moderate degree of polarity. If the K value is slightly off from the proper range, it can be adjusted by modifying the volume ratio. As a result, most of the unsuitable solvent systems are excluded for further study. With other considerations such as peak resolution and retention of the stationary phase, one can finally choose a suitable two-phase solvent system for their analytes.

RECENT APPLICATIONS

Isolation and Purification of Natural Products by CCC

One of the main areas of CCC application is in the isolation and purification of bioactive compounds from natural products. The CCC method provides an advantage over the conventional column chromatography by eliminating the

use of a solid support where an amount of stationary phase is limited, and dangers of irreversible adsorption from the support are inevitably present. Thus, both crude plant extracts and semipure fractions can be chromatographed, with sample loads ranging from milligrams to grams. Furthermore, the use of two-phase solvent systems allows one to choose solvents from an enormous number of possible combinations, which enables the CCC technique to separate compounds with a wide range of polarities. As a result, CCC comes to be a powerful chromatographic tool for preparative isolation and purification of bioactive compounds from natural sources. Moreover, the production of active compounds or fractions can be used as pure reference standards for further biological, pharmacological, and clinical studies. In this review, the work on plant constituents is summarized in Table 1 and some wonderful examples follow.

Flavonoids are one kind of important natural products. Recently, a preparative CCC was used to isolate and separate chemical constituents from the leaf of *Patrinia villosa*, a famous traditional Chinese medicinal herb.[19] Six flavonoids (compound 1–6, see Figure 2), including two known and four novel compounds, were successfully simultaneously purified by CCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (10 : 13 : 13 : 10, v/v) by increasing the flow rate of the mobile phase from 1.0 mL/min to 2.0 mL/min after 110 min, to bring out the late eluters. Among them, compounds **2**, **3**, **4**, and **5** were new compounds and discovered from nature for the first time. Moreover, their anticancer activities were also examined to inhibit human cancer cells' growth including A549, BEL-7402, SGC-7901, MCF-7, HT-29, K562, and A498 cell lines by the MTT method in vitro. The results indicated that the compounds **1**, **2**, and **3** exhibited high anticancer activities ($IC_{50} < 7$ μg/mL), especially to the K562 cancer cell ($IC_{50} < 3.1$ μg/mL), and the compounds **4**, **5,** and **6** exhibited weaker inhibition effects ($IC_{50} < 30$ μg/mL).

Studies on the CCC of bioactive triterpene saponins from *Momordica charantia* L have been performed on a Model GS10A HSCCC instrument (Beijing Institute of New Technology Application, Beijing, China).[20] Two fractions from silica gel column chromatography of the crude extracts were chromatographed with two biphasic solvent systems composed of methyl tert-butyl ether (MTBE)-n-butanol-methanol-water in the proportions of 1 : 2 : 1 : 5 and 1 : 3 : 1 : 5 (v/v), respectively. Four saponins, goyaglycoside-e, momordicoside L, goyaglycoside-a, and momordicoside K were obtained and confirmed by means of ESI-MS, ^{1}H- and ^{13}C-NMR.

Moreover, polyphenols provide a considerable separation challenge. The polarity and complexity of polyphenols is often a barrier to the elucidation of their structures and other characteristics. CCC offers an effective approach to deal with these problems.[21–23] Recently, polyphenols containing high molecular weight proanthocyanidins were separated and fractionated from the hop bract region (HBP) by CCC with the two-phase solvent system composed of MTBE: CH_3CN : 0.1% aqueous trifluoroacetic acid (2 : 2 : 3, v/v).[24]

Table 1. Separations of natural products by CCC

Sample	Solvent system (v/v)	Mobile phase	Ref.
Patrinia villosa Juss. flavonoids	n-Hexane-EtOAc-MeOH-H_2O (10 : 13 : 13 : 10)	Lower	[19]
Patrinia villosa Juss. flavonoids	n-Hexane-EtOAc-MeOH-H_2O (10 : 11 : 11 : 18)	Lower	[31]
Momordica Charantia L. *Triterpene saponins*	MTBE-n-BuOH-MeOH-H_2O (1 : 2 : 1 : 5)	Lower	[20]
Hop bract region polyphenols	MTBE-CH_3CN-0.1%TFA (2 : 2 : 3)	Upper	[24]
Polygonum cuspidatum Sieb. resveratrol, emodin, physcion	Light petroleum-EtOAc-MeOH-H_2O (3 : 5 : 4 : 6)-(3 : 5 : 7 : 3) in gradient	Lower	[29]
Patrinia villosa Juss Aurentiamide acetate	n-Hexane-EtOAc-MeOH-H_2O (1 : 1.2 : 1.2 : 1)	Lower	[34]
Rheum tanguticum Maxim. ex Balf. *trans*-3,5,4′-trihydroxystilbene-4/-*O*-β-D-glucopyranoside (+)catechin	EtOAc-EtOH-H_2O (25 : 1 : 25) (5 : 1 : 5)	Lower	[30]
Trollius ledebouri flavonoid glycosides	EtOAc-n-BuOH-H_2O (2 : 1 : 3)	Lower	[32]
Radix Isatis clemastanin B, indigoticoside A	EtOAc-n-BuOH-H_2O (2 : 7 : 9)	Lower	[33]
Tea cultivars catechin constituents TRI 2023 TRI 2025 TRI 2043 TRI 3079 TRI 4006	Hexane-EtOAc-MeOH-H_2O (1 : 6 : 1 : 6) (1 : 7 : 1 : 7) (1 : 7 : 1 : 7) (1 : 5 : 1 : 5) (1 : 6.5 : 1 : 6.5)	Lower	[35]
Forsythia suspensa phillyrin	n-Hexane-EtOAc-EtOH-H_2O (1 : 9 : 1 : 9)	Lower	[36]
*Schisandra chinens*is Schizandrin, gomisin A	n-Hexane-EtOAc-MeOH-H_2O (1 : 0.9 : 0.9 : 1)	Lower	[37]
Glycyrrhiza uralensis Risch. Liquiritigenin, isoliquiritigenin	n-Hexane-EtOAc-MeOH-CH_3CN-H_2O (2 : 2 : 1 : 0.6 : 2)	Lower	[38]
Smilax glabra rhizome Astilbin, isoastilbin	n-Hexane-n-BuOH-H_2O (1 : 1 : 2)	Lower	[39]
Acer truncatum Bunge. methyl gallate	EtOAc-EtOH-H_2O (5 : 1 : 5)	Lower	[40]
Artemisia rupestris L. rupestonic acid	n-Hexane-EtOAc-MeOH-H_2O (6 : 4 : 3.5 : 6.5)	Lower	[41]
Aucklandia lappa Decne costunolide dehydrocostuslactone	Light petroleum-MeOH-H_2O (5 : 6.5 : 3.5)	Lower	[42]

(*continued*)

Table 1. Continued

Sample	Solvent system (v/v)	Mobile phase	Ref.
P*eucedanum decursivum* (Miq.) maxim coumarin	Light petroleum-EtOAc-MeOH-H_2O (5 : 5 : 7 : 4)	Lower	[43]
Paeonia suffruticosa flavonoids	EtOAc-EtOH-HOAc-H_2O (4 : 1 : 0.25 : 5)	Lower	[44]
Spinach and sweet corn carotenoids	n-Hexane-EtOH-H_2O (6 : 5 : 1.3)	Lower	[45]
Evodia rutaecarpa (Juss.) Benth alkaloids	n-Hexane-EtOAc-MeOH-H_2O (5 : 5 : 7 : 5)	Lower	[46]
Scutellaria baicalensis baicalein wogonin oroxylin A	n-Hexane-EtOAc-n-BuOH-H_2O (1 : 1 : 8 : 10)	Lower	[47]
Grape seed flavan-3-ol phloroglucinol	n-Hexane-EtOAc-MeOH-H_2O (0.1 : 5 : 0.1 : 5) (1.5 : 10 : 1.5 : 10)	Lower	[48]
Cortex fraxinus coumarin	n-BuOH-MeOH-0.5%HOAc (5 : 1.5 : 5)	Lower	[49]
Acer truncatum Bunge quercetin-3-O-L-rhamnoside	EtOAc-EtOH-H_2O (5 : 1 : 5)	Lower	[50]
Curcuma wenyujin germacrone and curdione	Light petroleum-EtOH-Et_2O-H_2O (5 : 4 : 0.5 : 1)	Lower	[51]
Scutellaria baicalensis Georgi baicalin and wogonoside	EtOAc-MeOH-1%HOAc (5 : 0.5 : 5)	Lower	[52]
Schisandra chinensis (Turcz.) baill deoxyschisandrin and r-schisandrin	n-Hexane-MeOH-H_2O (35 : 30 : 3)	Lower	[53]
Schisandra chinensis (Turcz.) baill *microalga microcystis aeruginosa* bioactive carotenoid zeaxanthin	n-Hexane-EtOAc-EtOH-H_2O (8 : 2 : 7 : 3)	Lower	[54]
Schisandra Chinensis (Turcz) Baill schisandrin and schisantherin	n-Hexane-EtOAc-MeOH-H_2O (22 : 8 : 20 : 20)	Lower	[63]
Epimedium koreamum Nakai flavonoids	$CHCl_3$-MeOH-H_2O (4 : 3.5 : 2)	Lower	[55]
Fructus Arctii. arctiin	EtOAc-n-BuOH-EtOH-H_2O (5 : 0.5 : 1 : 5)	Upper	[56]
Plantago psyllium L. acteoside and isoacteoside	EtOAc-H_2O (1 : 1)	Lower	[57]
Zingiber cassumunar phenylbutenoids	Light petroleum–ethanol–diethyl ether–H_2O (5 : 4 : 2 : 1)	Lower	[58]
Tea tea catechins and polyphenols	MTBE-CH_3CN-0.1% TFA (2 : 2 : 3)	Upper	[59]

(*continued*)

Table 1. Continued

Sample	Solvent system (v/v)	Mobile phase	Ref.
Salvia miltiorrhiza Bunge salvianolic acids	n-Hexane-EtOAc-MeOH-H_2O (1.5 : 5 : 1.5 : 5)	Lower	[60]
Rabdosi rubescens oridonin	n-Hexane-EtOAc-MeOH-H_2O (1 : 2 : 1 : 2)	Lower	[61]
Corydalis yanhusuo alkaloids	CCl_4-$CHCl_3$-MeOH-0.2 M HCl (1 : 7 : 3 : 4) & $CHCl_3$-MeOH-0.2M HCl (7 : 3 : 4)	Lower	[62]
Stachytarpheta cayennensis (Rich.) Vahl Phenylpropanoid, iridoid glycosides	EtOAc-n-BuOH-H_2O (1 : X : 1)	Lower	[64]
Siparuna guianensis free and glycosylated flavonoids	n-Hexane-EtOAc-MeOH-H_2O (0.6 : 4.0 : 0.05 : 1.0) (0.6 : 4.0 : 0.7 : 1.0)	Lower	[65]
Aloe vera minor active chromone	n-Hexane-EtOAc-MeOH-H_2O (1 : 5 : 1 : 5)	Lower	[66]
Cecropia lyratiloba Miquel triterpene	Hexane-EtOAc-MeOH-H_2O (1 : 2 : X : 1)	Lower	[67]
Callus culture rosmarinic acid	$CHCl_3$-n-BuOH-H_2O (4.5 : 1 : 4.5)	Lower	[68]
Tripterygium wilfordii Hook F. tripdiolide	n-Hexane-CH_2Cl_2-H_2O (3 : 22 : 17 : 8) & CH_3Cl-MeOH-H_2O (4 : 3 : 2)	Lower	[69]
Kava root kavalactones	n-Hexane-EtOAc (1 : 1)	Lower	[70]
Polygala tenuifolia scrose esters	$CHCl_3$-MeOH-H_2O (3 : 3.5 : 2) EtOAc-n-BuOH-EtOH-H_2O (4 : 0.6 : 0.6 : 5)	Lower	[71]
Benincasa hispida phenolic compounds	n-Hexane-n-BuOH-MeOH-H_2O (10 : 16 : 5 : 20) n-Hexane-EtOAc-MeOH-H_2O (1 : 1 : 1 : 1)	Lower	[72]
Hypericum japonicum thumb. flavonoids, phloroglucinol	EtOAc-MeOH-H_2O (5 : 1 : 5) n-Hexane-EtOAc-MeOH-H_2O (1 : 1.2 : 1.2 : 1)	Lower	[73]
Edgeworthia chrysantha Lindl coumarins	n-Hexane-EtOAc-MeOH-H_2O (4 : 6 : 4 : 6)	Lower	[74]
Prunus armeniaca L. amygdalin	n-BuOH-EtOAc-H_2O (4 : 1 : 6)	Lower	[75]
Sophora flavescens flavonones	n-Hexane-EtOAc-MeOH-H_2O (1 : 1 : 1 : 1)	Lower	[76]

(*continued*)

Table 1. Continued

Sample	Solvent system (v/v)	Mobile phase	Ref.
Diospyros kaki barbinervic acid, rotungenic acid	n-Hexane-EtOAc-MeOH-H_2O (3 : 6 : 4 : 2)	Lower	[77]
Radix saposhnikoviae prim-O-glucosyl-cinmifugin 4′-O-β-D-glucosyl-5-O-methylvisamminol	$CHCl_3$-MeOH-H_2O (10 : 8 : 4)	Lower	[78]
Ocotea elegans neolignans	n-Hexane-EtOAc-MeOH-H_2O (1 : 2 : 2 : 1)	Lower	[79]
Radix linderae linderalactone lindenenol	Light petroleum-EtOAc-MeOH-H_2O (5 : 5 : 6 : 4)	Lower	[80]

CCC is very effective for initial fractionation or purification of crude plant extracts. It can be used for all ranges of polarities but has special advantages for the handling of polar extracts, which are often difficult to process by more classical techniques. A hydrophilic organic/salt containing an aqueous

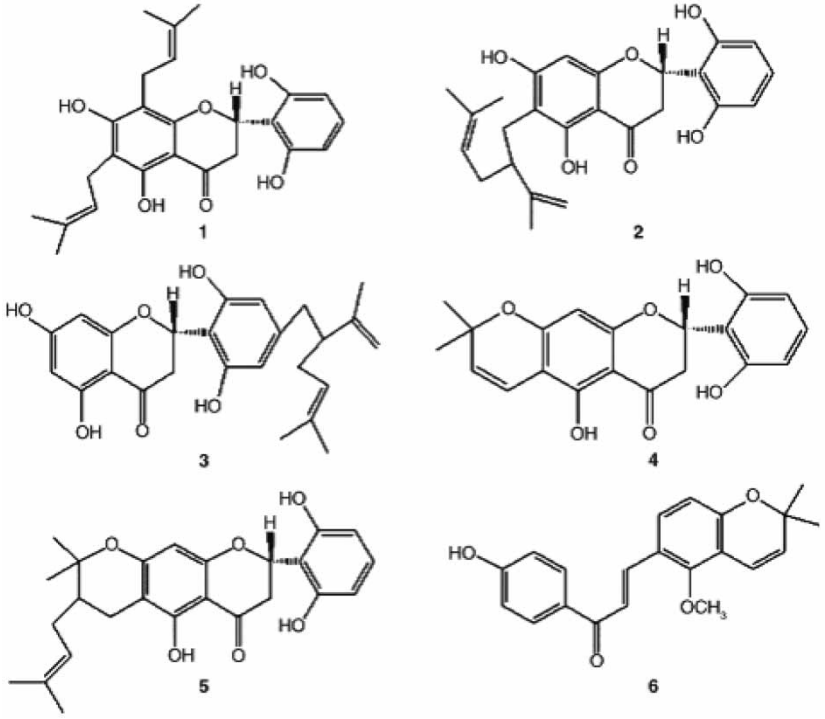

Figure 2. Chemical structures of the isolated flavonoids. (Reprinted from ref. 19 with permission).

two-phase system was recently established by Zhi et al., and salvianolic acid B was successfully isolated from the crude extract of *Salvia miltiorrhiza* by CCC with this system.[25] Ethanol and n-propanol were selected to constitute biphasic systems with ammonia sulphate, sodium chloride, and phosphate separately, and related system characteristics including phase diagrams, phase ratio, separation time were tested. The partition coefficient of crude salvianolic acid B was also tested in the above systems and further finely adjusted by altering the constitution of phosphate in a n-propanol/phosphate system. Salvianolic acid B was purified to 95.5% purity by CCC in a 36% (w/w) n-propanol/8% (w/w) phosphate system, with the ratio between dipotassium hydrogen phosphate and sodium dihydrogen phosphate of 94 : 6. One hundred and eight milligrams of salvianolic acid B was purified from 285 mg crude extract with the recovery of 89%.

In addition, the scaling-up of CCC for industrial use is very promising and challenging, though HSCCC is intensively used in preparative separation in laboratories. One way to scale up CCC is to utilize the slow rotary mode of coiled column, which was first described by Ito and Bhatnagar.[26,27] In this system, the best result was attained by rotating the coil slowly around its horizontal axis at a critical speed that yields high retention of the stationary phase. Recently, an apparatus called slow rotary CCC (SRCCC) equipped with a 40-L capacity column made of 17 mm I.D. (Figure 3) convoluted tubing was reported by Du et al.[28] Using this apparatus, a 500 g amount of crude *Salix alba* extract containing salicin at 13.5% was separated yielding 63.5 g of

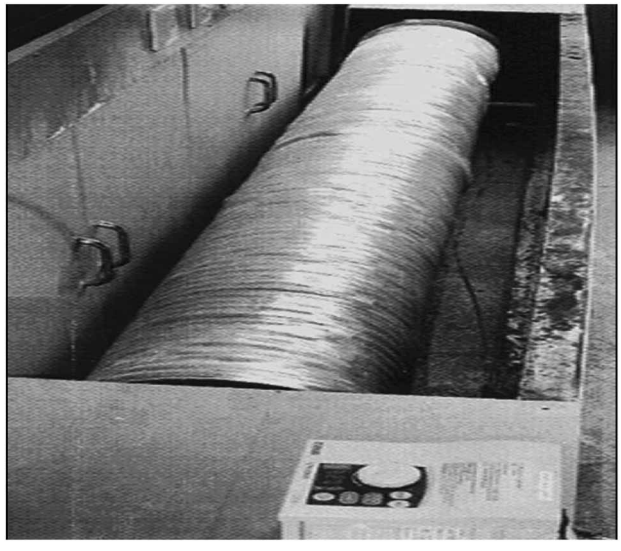

Figure 3. Photograph of our slow rotary countercurrent chromatograph equipped with a pair of rotary seals and a 40 L column. (Reprinted from ref. 28 with permission).

salicin at 95.3% purity in 20 h, using MTBE–n-butanol (1 : 3, v/v) saturated by methanol–water (1 : 5, v/v) as a stationary phase and methanol–water (1 : 5, v/v) saturated by MTBE–n-butanol (1 : 3, v/v) as a mobile phase. The flow rate of the mobile phase was 50 mL/min. Similarly, a 400 g amount of crude *Semen armeniacae* extract containing amygdalin at 55.3% was also isolated to yield 221.2 g of amygdalin at 94.1% purity in 19 h using ethyl acetate–n-butanol (1 : 2, v/v) saturated by water as a stationary phase and water saturated by ethyl acetate–n-butanol (1 : 2, v/v) as a mobile phase.

Elution Modes in CCC

Although, CCC is a very effective tool for the preparative separation and purification of natural products, the extracts from plant materials usually contain a high number of different compounds with a broad range of hydrophobicity. Most often, only one component could be separated from the others using a single solvent system. In order to separate more different hydrophobic compounds and shorten the separation time, scientists applied different elution modes in CCC methodology, such as stepwise elution, gradient elution, extrusion elution, and the current method.

Stepwise Elution

A stepwise elution mode was employed for isolation and purification of *trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-d-glucopyranoside (compound I) and (+) catechin (compound II) from *Rheum tanguticum* Maxim. ex Balf. extract.[30] For the first 5 hours of chromatography, the solvent system was ethyl acetate-ethanol-water (25 : 1 : 25, v/v; lower phase as mobile phase), modified to the proportions 5 : 1 : 5 for the next 6 hours, and the flow rate of the mobile phase was increased from 0.8 mL/min to 2.0 mL/min. The CCC separation was performed on 250 mg of crude extract yielding pure compound I (10.2 mg) and compound II (26.7 mg), all at purities of over 96% in a single run (Figure 4).

Gradient Elution

Hydroxyanthraquinones from *Rheum officinale* (Polygonaceae) had been separated by using pH-gradient elution. Diethyl ether and 1% NaH_2PO_4 were pumped simultaneously into the coils of a TBE-300A chromatograph to give a volume ratio of 40 : 60, respectively. After injection of the crude anthraquinone sample, elution was begun with 1% NaH_2PO_4 as mobile phase. A linear gradient of 1% NaH_2PO_4 and 1% NaOH (100 : 0 to 0 : 100 over 500 min) was then run (Figure 5). By this means, five pure hydroxyanthraquinones were obtained.[81]

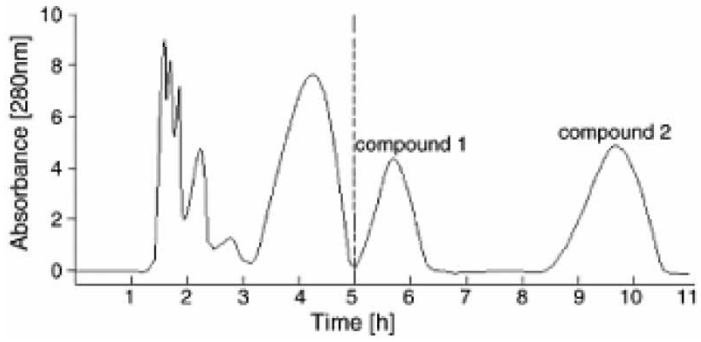

Figure 4. HSCCC of fraction of the 95% EtOH extract of *R. tanguticum.* solvent system: ethyl acetate-ethanol-water (25 : 1 : 25, v/v) and (5 : 1 : 5, v/v), stationary phase: upper organic phase of ethyl acetate-ethanol-water (25 : 1 : 25, v/v); mobile phase: lower aqueous phase of ethyl acetate-ethanol-water (25 : 1 : 25, v/v) before 5 h and ethyl acetate-ethanol-water (5 : 1 : 5, v/v) after 5 h; flow-rate: 0.8 mL/min before 5 h and 2 mL/min after 5 h, revolution speed: 800 rpm; sample: 250 mg dissolved in 10 mL lower phase; retention of the stationary phase: about 53%. (Reprinted from ref. 30 with permission).

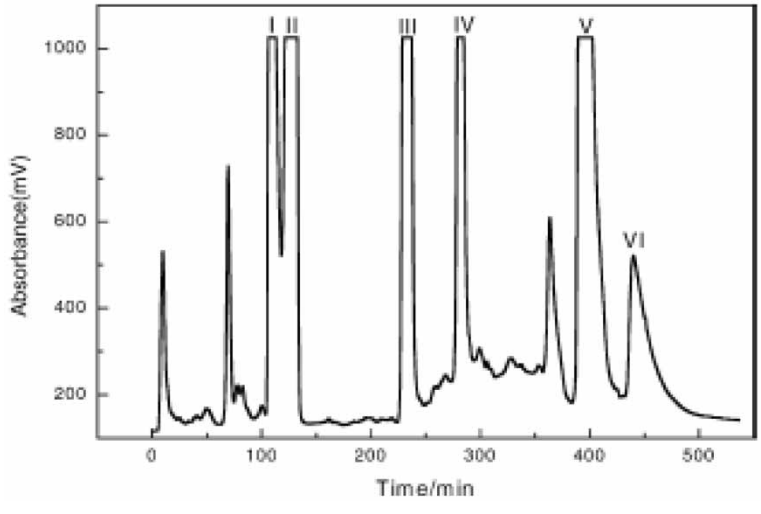

Figure 5. HSCCC chromatogram of crude extract from *R. officinale* Baill. Stationary phase: aether; mobile phase: 1% NaH_2PO_4 and 1% NaOH to perform pH-gradient elution (1% NaH_2PO_4 : 1% NaOH = 100 : 0–0 : 100 in 500 min); flow rate: 2.0 mL/min; revolution speed: 800 rpm; sample size: 120 mg crude extract dissolved in 20 mL of aether; temperature; retention of the stationary phase: 40%. I: Rhein; II: cinnamic acid; III: emodin; IV: aloe-emodin; V: chrysophanol; VI: physcion. (Reprinted from ref. 81 with permission).

Elution-Extrusion Method

The elution-extrusion procedure established by Berthod et al.[82,83] is an effective way to avoid any irreversible adsorption of solutes in the column. The method relies on the fact that, the liquid volumes occupied by the solutes highly retained inside the column can be orders of magnitude lower than the mobile-phase volume that would be needed to elute them. The elution-extrusion method has two steps: the first step is a regular CCC chromatogram. Next, the stationary phase containing the partially separated hydrophobic solutes is extruded out of the column in a continuous way using the liquid stationary phase. Berthod used alkylbenzene homologues as model compounds with the heptane/methanol/water biphasic liquid system to establish the theoretical treatment and compare the performance of two types, hydrodynamic and hydrostatic, of CCC columns (Figure 6). The results show that the method can dramatically boost the separation power of the CCC technique. An apparent efficiency higher than 20000 plates was obtained for extruded octylbenzene and a 160-mL hydrodynamic CCC column with less than 500 plates when conventionally used.

Cocurrent Method

The cocurrent method relies on the fact that the stationary phase is a liquid.[84] It is possible to push it slowly in the same direction as the mobile phase. The result will be that no compound can be trapped inside the column. The most retained compound sticks to the stationary phase. It will eventually elute since the stationary phase slowly moves toward the column exit. This method can be also described as a "truly" moving bed chromatography, since the stationary phase is slowly moving in the same direction as the mobile phase. This could be a first step in using the liquid character of the stationary phase in view of continuous separations following the "simulated" moving bed (SMB) method with classical solid stationary phase. The theoretical foundation was summarized by Berthod et al., and a mixture of five steroid compounds of widely differing polarities was used as a test mixture to evaluate the capabilities of the method with the biphasic liquid system made of water/methanol/ethyl acetate/heptane 6/5/6/5 (v/v) and a 53 mL CCC column of the coil planet centrifuge type (Figure 7). The results show that the chromatographic resolution obtained in the cocurrent method was very good because the solute band broadening was minimized as long as the solute was located inside the "stationary" phase. Pushing the method at its limits, it was demonstrated that the five steroids could still be (partly) separated when the flow rate of the two liquid phases was the same (2 mL/min). This was due to the higher volume of upper phase (72% of the column volume) contained inside the cocurrent column, producing a lower linear speed compared to the aqueous lower phase linear speed. The capabilities of the cocurrent method compare well with those of

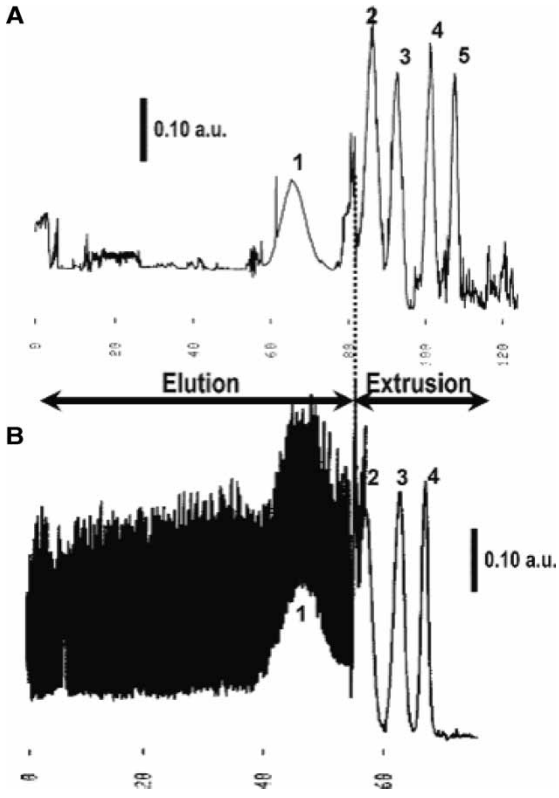

Figure 6. Elution-extrusion method. (A) Chromatogram obtained with a hydrodynamic machine. Flow rate, 4 mL/min polar phase in the head-to-tail direction during the elution step (85 min), switched to heptane used as the extruding agent in the head-to-tail direction (35 min), 800 rpm. Peak identification: (1) benzene, (2) toluene, (3) ethylbenzene, (4) butylbenzene, and (5) octylbenzene; injection volume, 1 mL in heptane. (B) Chromatogram obtained with a hydrostatic CPC machine. Flow rate, 4 mL/min with polar phase (descending, head-to-tail direction, elution step, 55 min) and heptane (same direction, extrusion step, 20 min), 1000 rpm. Peak identification: (1) benzene, (2) toluene, (3) butylbenzene, and (4) octylbenzene; injection volume, 1 mL in heptane. Detection, UV at 254 nm. (Reprinted from ref. 82 with permission).

the gradient elution method in HPLC. However, continuous detection was a problem due to the fact that two immiscible liquid phases eluted from the column. It could be partly solved using an evaporative light scattering detector.

Multidimensional CCC

In CCC separation, when two peaks overlap, it is a common practice that each peak fraction is pooled, dried, and rechromatographed with the same or a

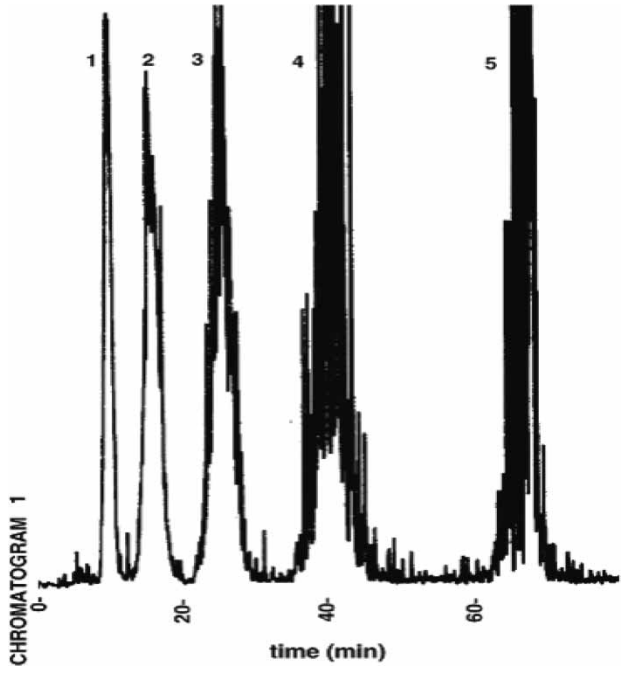

Figure 7. Actual chromatogram of the separation of 5 steroids by cocurrent CCC. Liquid system: water/methanol/ethyl acetate/heptane 6/5/6/5 (v/v). Mobile phase: lower aqueous phase, flow rate 2 mL/min; "stationary" phase: upper phase at 0.5 mL/min flow rate. Machine volume $VC = 53$ mL. Rotor rotationspeed: 800 rpm. Detection ELSD. The peak order is: (1) prednisone(0.32 mg); (2) prednisolone acetate (0.34 mg); (3) testosterone (0.42 mg); (4) estrone (1.5 mg) and (5) cholesterol (1.1 mg). Injection volume 200 μL of the steroids in lower phase. (Reprinted from ref. 84 with permission).

slightly modified solvent system to improve the yield of pure fraction. This is possible because the yield of a target compound in CCC depends on the amount of the impurities in the fraction, i.e., the smaller the amount of impurity, the higher the yield of target compound. Of course, purification of the partially resolved two compounds in this method each requires another individual run and separation time. However, Yang et al. developed a multi-dimensional CCC (MDCCC) method for simultaneous separation of three flavone aglycones.[85] This MDCCC system (Figure 8) was used with two same multilayer coil planet centrifuges. Two constant flow pumps were used to elute the mobile phase while continuous monitoring of the effluent was achieved with two UV detectors at 254 nm. Two manual six-port valves, one with a 20 mL loop used as the injection valve and the other without a loop used as the switching valve were used to introduce the sample into the column. This method improved both yield and separation time by introducing

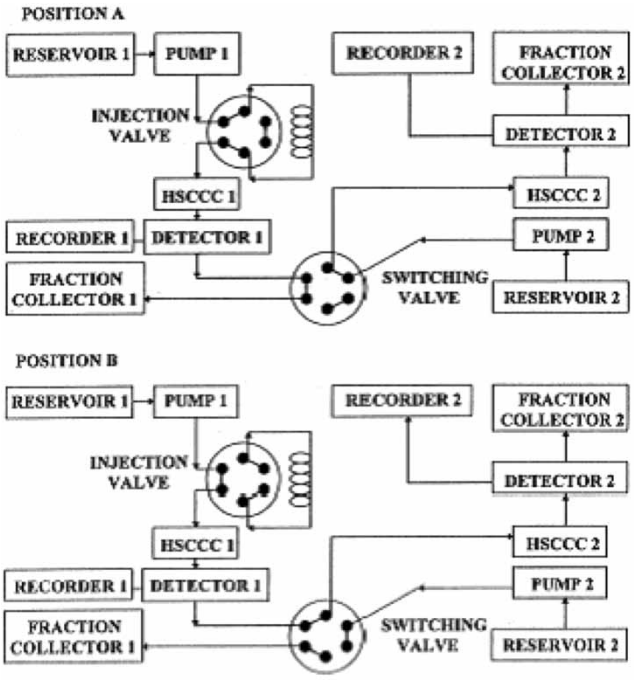

Figure 8. Schematic diagram of our multidimensional countercurrent chromatography. The effluent from the outlet of HSCCC 1 is rent chromatography (MDCCC) system with two sets of high-speed countercurrent chromatography (HSCCC) systems, a six-port injection valve and a six-port switching valve. (Reprinted from ref. 85 with permission).

the first peak into the second column to separate it in tandem. In addition, the cut and introduced portion of the peak into the second column will yield improved peak resolution. Recently, the MDCCC method was successfully applied to isolation and purification of imperatorin, oxypeucedanin, and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook with a pair of two-phase solvent systems composed of *n*-hexane-ethyl acetate-methanol-water at volume ratios of 1 : 1 : 1 : 1 (v/v) and 5 : 5 : 4.5 : 5.5 (v/v).[86] Similarly, tripdiolide was also isolated with over 98% purity from *Tripterygium wilfordii* Hook F. by the MDCCC method using *n*-hexane-dichloromethanemethanol-water (3 : 22 : 17:8, v/v) and chloroform-methanol-water (4 : 3 : 2, v/v) as the pair of two-phase solvent systems.[87]

pH-Zone-Refining CCC

pH-zone-refining CCC, which was developed by Ito,[88,89] is generally employed as a large-scale preparative technique for separating ionizable analytes. The

mechanism of pH-zone-refining CCC were described in several monographs and review articles. This method elutes highly concentrated rectangular peaks fused together with minimum overlapping, while impurities are concentrated and eluted between outside the major peaks according to their p*K*a and hydrophobicity. The greatest advantage of the method is its large sample loading capacity, which exceeds 10-fold that of the standard HSCCC in the same separation column. In addition, the method provides various special features such as yielding highly concentrated fractions, concentrating minor impurities for detection, and allowing the separation to be monitored by the pH of the effluent when there are no chromophores. Since the analytes are ionizable compounds, most separations can be performed using a relatively polar solvent system. Furthermore, selection of solvent systems and preparation of the sample are quite different from those used in the standard HSCCC technique.

Recently, pH-zone-refining CCC was successfully applied to the separation of cichoric acid from *Echinacea Purpurea* (L.) Moench.[90] A 3.0 g quantity of sample was separated using the following two-phase solvent system: MTBE–acetonitrile–water (4 : 1 : 5, v/v), 10 Mm trifluoroacetic acid in organic stationary phase and 10 mM ammonia in aqueous mobile phase. Similarly, three alkaloids were isolated and purified from 3.1 g of the crude extract of *Corydalis decumbens* (Thunb.) by this method with a two-phase solvent system composed of MTBE-acetonitrile-water (2 : 2 : 3, v/v), where triethylamine (5–10 mM) was added to the upper organic stationary phase as a retainer and hydrochloric acid (5–10 mM) to the aqueous mobile phase as an eluter.[91] In addition, the *Picralima* alkaloids and natural coloring agents, Kaoliang and Lac colors, were also successfully isolated and purified by pH-zone-refining CCC.[92,93]

Bioseparation by CCC

CCC has the unique features of high recovery, high efficiency, and the ease to scale-up, and has been widely used in the separation and purification of natural products. However, CCC is also an effective chromatographic approach for the purification of biological macromolecules such as proteins, nucleic acids, and cells, with the aqueous polymer two-phase systems.[94,95] The combination of the aqueous polymer two-phase system with CCC, could resolve the limitations of aqueous polymer two-phase system extraction, including low efficiency in single-step operation and difficulties in performing continuous extraction. However, the high viscosity and low interfacial tension of aqueous polymer two-phase systems tend to cause emulsification of the two phases, and results in lower and unstable retention of stationary phase in the conventional type-J CCC apparatus. Hence, a series of CCC apparatus suitable for aqueous polymer two-phase systems was designed by Ito and coworkers, including cross-axis coil planet centrifuge, nonsynchronous coil planet centrifuge, and spiral disk assembly fitted on synchronous coil planet centrifuge, among

which, the cross-axis coil planet centrifuge has better balance between the complexity of structure and the separation efficiency, and have been used for the separation and purification of a variety of proteins.[96]

Recently, a novel self-designed HSCCC apparatus, model TBE-300V, has been fabricated by Tauto Biotech,[97] which is a vertical multicolumn synchronous CCC apparatus with features of simplified structure, self balance without counterweight, controllable temperature, and the ease to scale-up (Figure 9). This instrument was applied for purification of α-amylase from the cultivation supernatant of recombinant *Bacillus subtil*. α-Amylase is a commercially important enzyme which is widely used in many industrial fields, such as starch processing, brewage, sugar refining, textile treatment, detergent, and fermentation.[98] The conventional purification procedures of α-amylase include precipitation with ammonia sulphate, absorbtion with starch, ion-exchange chromatography, and size exclusion chromatography. The total recovery of α-amylase after the above steps is only around 40%. With this TBE-300V instrument, PEG4000-citrate aqueous polymer two-phase system containing 2% (w/w) sodium chloride and supplemented with 0.56% (w/w) $CaCl_2$ as protective agent was successfully applied to purify α-amylase from cultivation supernatant to homogeneity, and significantly increased the recovery of purification.

In addition, a new small scale *X*-axis CPC (Figure 10) was designed and fabricated by Shinomiya et al.[99] Performance of this apparatus was evaluated on protein separation using an aqueous–aqueous polymer phase system composed of polyethylene glycol 1000 and dibasic potassium phosphate with four multilayer coiled columns. A series of experiments revealed that the combination of right- and left-handed coils produced the best partition efficiencies for both lower and upper mobile phases by selecting the revolution direction.

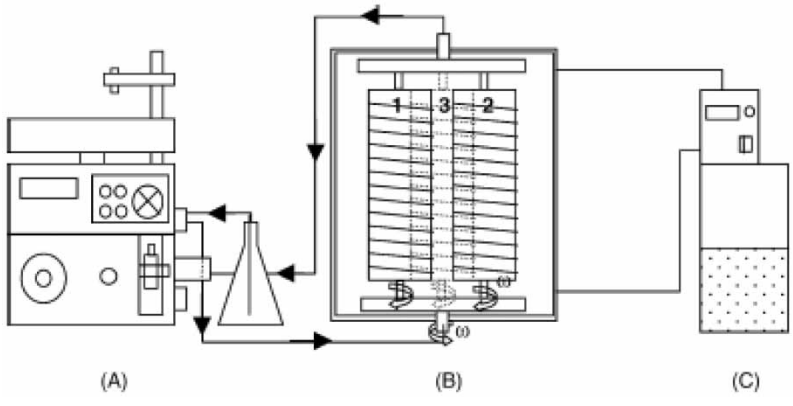

Figure 9. Diagram of HSCCC, model TBE-300 V. (A) Akta Prime; (B) TBE-300 V; (C) water bath; (1–3) coiled separation columns. (Reprinted from ref. 97 with permission).

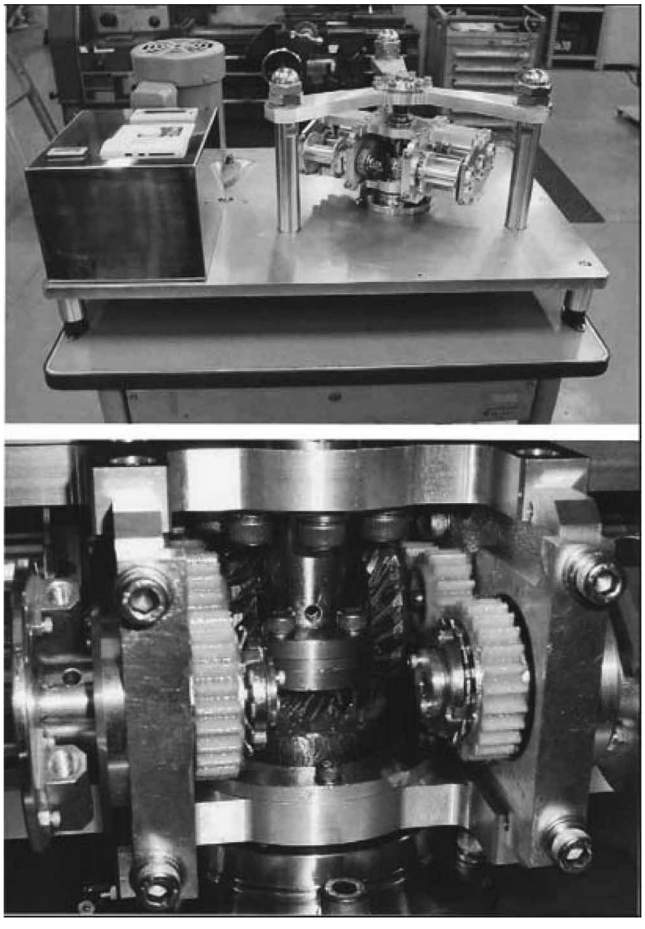

Figure 10. Overview of the present *X*-axis CPC without the cover case (the upper photograph) and the drive units (the lower photograph). (Reprinted from ref. 99 with permission).

The overall results indicate that the head–tail elution mode substantially affects the peak resolution and stationary phase retention. This new type of CCC combines the right- and left-handed coils and gives good partition efficiencies regardless of the choice of the mobile phase. Therefore, it will be useful for the separation of various kinds of biologically active compounds.

Enantioseparation by CCC

The CCC method for the enantiomeric separations were reviewed by Foucault,[100] with discussions of the basic theory of chiral CCC; and the

original used three kinds of chiral selectors (CSs). Separation of enantiomers by CCC involves the addition of a suitable chiral CS to one of the phases of the biphasic solvent system used. The phase containing the CS is used as stationary phase. To obtain successful chiral separations, the ideal solvent system should meet certain requirements. Thus, the leakage of CS from one phase to the other should be avoided while promoting the desired partition of the analyte enantiomers between the two liquid phases. Moreover, the solubility of the CS in the stationary phase has to be sufficient to bring about the separation and the elution of the analytes in a reasonable amount and time for preparative purposes. The CSs employed in CCC until the present were, in general, those directly used also in CE, HPLC, and in other separation techniques. Although, it is difficult to find a suitable CS (being highly selective for the given racemic compounds) and the appropriate system of solvents, with the improvements in the instrumentation and further optimizations of the different parameters involved in the overall separation process, this has been, in general, overcome in the most recent applications.

L-Proline Derivatives

N-Dodecanoyl-L-proline-3,5-dimethylanilide was then used in an authoritative way by Ito et al. to perform complete analytical studies and introduce the pH-zone-refining mode of CCC for chiral discrimination.[101–103] Recently, several L-proline and (4*R*)-hydroxy-l-proline derivatives were evaluated as CSs in the separation of enantiomers by CCC. A variety of biphasic solvent systems, all of organic/aqueous nature, and the pH of the buffer solutions were tested in order to determine the appropriate distribution for CSs and the racemates. Successful separations of DNB- ($\pm$)-leucine in analogous experimental conditions allow the comparative study of the enantioselectivity displayed by the considered CSs.[104]

Carboxymethyl β-Cyclodextrin

CCC was successfully used for enantioseparation of chlorpheniramine using Carboxymethyl β-cyclodextrin as the CS.[105] The separation was performed with a two-phase system composed of ethyl acetate:methanol:water (10 : 1 : 9, v/v) in a tail-to-head elution mode. The lower phase was used as the stationary phase and contained 20 mmol/L of the CS. Within 2 hours, 3 mg of racemic chlorpheniramine was isolated in a single CCC run. Similarly, enantioseparation of aminoglutethimide was also performed by HSCCC with the same two-phase system. The lower phase contained 20 mmol/L of carboxymethly-β-cyclodextrin as chiral selector and was used as the stationary phase. The enantiomers were separated in 1.2 h and identified by chiral HPLC.[106]

(+)-(18-Crown-6)-Tetracarboxylic Acid

(+)-(18-Crown-6)-tetracarboxylic acid ($18C6H_4$) has been known as a highly efficient chiral selector for resolving primary amine enantiomers in capillary electrophoresis (CE). Chung et al. investigated the chiral separation of gemifloxacin using $18C6H_4$ in analytical CCC. A successful separation of gemifloxacin enantiomers could be achieved using a two-phase solvent system composed of n-butanol-ethyl acetate-bis(2-hydroxyethyl)aminotris (hydroxymethyl)methane acetate buffer with a small amount of $18C6H_4$.[107]

Cellulose-Type CSs

The applicability of cellulose and amylose tris(3,5-dimethylphenylcarbamate) as CSs for the separation of enantiomers by CCC was investigated by Minguillon et al.[108] Partial enantioseparation of pindolol and warfarin could be achieved in methyl isobutyl ketone (MIBK)/aqueous solution and MTBE/aqueous solution, respectively. For these two racemates, enantiomeric excess values from 84% to 97% were achieved under the best conditions tested. In addition, cellulose was chemically modified with hydrophobic dodecanoyl groups followed by 3,5-dimethylphenylcarbamoyl substituents forming mixed ester/carbamate derivatives to improve the solubility in lipophilic solvents. In the classical elution mode and the pH-zone-refining displacement mode, which were applied, the enantioseparation of pindolol and warfarin was achieved with ethyl acetate and aqueous ammonium acetate or sodium phosphate buffer as two-phase solvent system.[109]

Cinchona Alkaloid Derivatives

Cinchona-derived anion-exchange-type CS was employed in CCC for separation of enantiomers of N-derivatized amino acids and 2-aryloxypropionic acids. The solvent systems used were composed of ammonium acetate buffer/*tert*-amyl alcohol/methanol/heptane and, especially, ammonium acetate buffer/MIBK or diisopropyl ether. Up to 300 mg of *N*-(3,5-dinitrobenzoyl)-(±)-leucine was totally resolved in a single run using a 10 mM concentration of CS in 122 mL of stationary phase. This amount could be increased up to 900 mg when the pH-zone-refining mode was applied.[110] In addition, a purposefully designed, highly enantioselective chiral stationary-phase additive (CSPA) derived from bis-1,4-(dihydroquinidinyl)phthalazine was developed by the same group for preparative enantiomer CCC separation of the herbicidal agent 2-(2,4-dichlorphenoxy)propionic acid (dichlorprop). With a solvent system consisting of 10 mM CSPA in MTBE and 100 mM sodium phosphate buffer (pH 8.0), 366 mg of racemic dichlorprop could be achieved.[111]

On-line Monitoring Methods

Generally, the effluent from the outlet of the CCC column may be continuously monitored by a UV–vis detector, as in conventional liquid chromatography. Furthermore, interfacing CCC with mass spectrometry would seem an ideal way of combining the separation capabilities of the chromatographic method with the excellent sensitivity and specific detection of MS. However, there were initially some difficulties with this hyphenated technique: (1) high back pressure of CCC; (2) suitable solvent system for both CCC and MS. During the past 10 years, considerable effort has been made to develop analytical HSCCC for interfacing mass spectrometry and CCC-TSP-MS has been successfully applied mainly to the separation of natural products, including the analyses of alkaloids, triterponic acids, and ligans. Recently, a 4.6 mL rotating coil instrument ("Milli-CCC") with 0.76 mm bore stainless steel tubing, which provides separations of samples within 5 min, was directly interfaced with ESI and APCI mass spectrometry. This method was developed for the isolation and purification of three flavonoids from the seeds of *Oroxylum indicum* (Bignoniaceae). Best results were obtained with a rotation speed of 1100 rpm and the solvent system hexane-ethyl acetate-methanol-0.2% formic acid 1 : 1.2 : 1 : 1 (v/v, lower phase as mobile phase), at a flow rate of 1 mL/min. With ESI, a split in the flow of eluent was necessary but with APCI no splitting was required.[112]

In addition, Zhou et al. reported a preparative isolation-purity detection hyphenated system: online coupling of HSCCC with HPLC–DAD[113] (Figure 11). The introduction of online purity analysis in HSCCC dramatically improved the efficiency of this technique by overcoming the drawbacks of post analysis in HSCCC isolation. In this system, the effluent from the outlet of HSCCC was split into two parts: one was collected, while the other was introduced directly into an HPLC–DAD system for purity analysis through a switch valve. Therefore, the purities of the obtained fractions from HSCCC were monitored, and fractions with high purities were collected. This strategy was successfully demonstrated with the preparative isolation and purification of hyperoside from *Hypericum perforatum*.

CONCLUSION

CCC provides a useful complementary technique to the conventional liquid chromatographic method in separation science. The methodology has been successfully utilized for routine use of the isolation and purification of natural products. Different elution modes, multidimensional methods, and pH-zone-refining techniques could be applied to CCC to enhance its separation efficiency. Moreover, CCC is also an effective chromatographic approach for the purification of biological macromolecules with the aqueous polymer two-phase systems. In addition, with the development of new

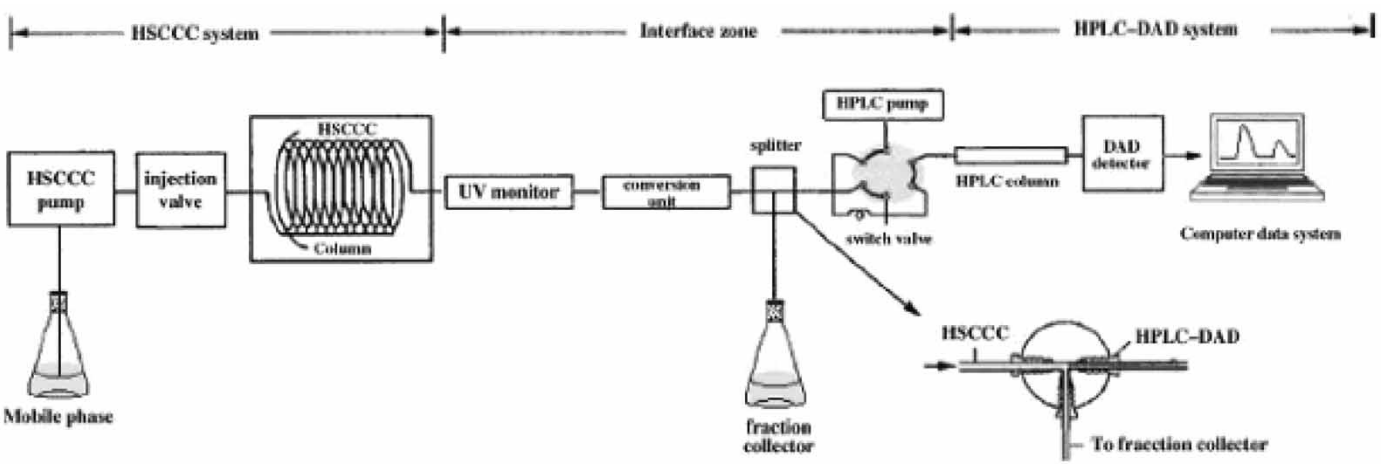

Figure 11. Schematic diagram of the hyphenated HSCCC–HPLC–DAD system and design of T-split. (Reprinted from ref. 113 with permission).

chiral selectors and suitable solvent systems, CCC has provided a suitable alternative technique in the field of chiral separation. Review of the current literature has demonstrated that CCC is an excellent approach to circumvent the problems associated with solid-phase adsorbents and will be a more promising separation method in the future.

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