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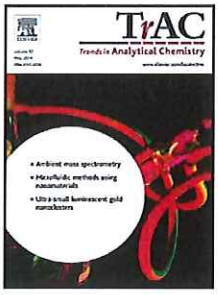

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An overview of recent progress in elution mode of counter-current chromatography

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Highlights

Overview of recent progress in elution mode of CCC.

We summarized the major benefits and limitations of different elution modes of CCC.

We described some novel elution modes developed for separation by CCC.

We discussed the challenges of different elution modes of CCC applied to real samples.

ABSTRACT: Counter-current chromatography (CCC) is a developing chromatographic technique which achieves the separation depending on the distribution of the target in immiscible biphasic or multiphasic solvent system. In past decades, this technique has made great progress in application and theory. This overview is mainly focused on the development of elution modes which can easily achieved on the classical CCC apparatus in recent years. It includes gradient elution, dual-mode elution, multiple dual-mode elution, recycling elution, extrusion elution, cocurrent elution and pH-zone refining. The basic principles of each elution mode are detailed described and summarized. Meanwhile, the contrast and the scope of application of these elution modes were also ~~be~~ discussed.

Keywords: Counter-current chromatography; Elution mode; Gradient elution; Dual-mode elution; Multiple dual-mode elution; Recycling elution; Extrusion elution; Cocurrent elution; pH-zone refining CCC

Abbreviations: CCC, counter-current chromatography; CPC, centrifugal partition chromatography; HSCCC, high-speed counter-current chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography;

1. Introduction

Counter-current chromatography (CCC) is a chromatographic separation and preparation technology which based on the liquid-liquid partition coefficient of the solute since no adsorptive matrix is employed to retain the stationary phase [1]. The liquid stationary phase is retained in the column by a centrifugal force field while the immiscible mobile phase passes through. Because its free solid stationary phase and continuous liquid-liquid partition design, CCC has many distinctive advantages compared with conventional chromatography techniques. CCC can avoid the sample loss caused by irreversible adsorption and solute degeneration caused by surface chemistry. It can be directly applied to crude extract and has sustained high efficiency, high recovery and low solvent consumption as well as ability for preparation of a large amount of compound. In addition, CCC can be easily coupled with other on-line separation techniques [2-4]. So, CCC has been found an increasingly wide application in many fields. Meanwhile, in separation of some special compounds, such as high polar compounds and unstable compounds, CCC displays unique advantage and great application potential. Now it has become a novel, worldwide separation and purification technique.

It should be pointed out that the CCC here is in a broad sense and it includes two main different types: one is hydrostatic centrifugal partition chromatography (CPC) which is based on a constant centrifugal force field; the other is hydrodynamic coil planet centrifuge which is based on a variable centrifugal force field and it be called high-speed CCC (HSCCC). CCC is a powerful and effective preparative technique due to its high capacity and low cost of solvent. In a CCC separation, the selection of solvent system has been considered the first and crucial factor because this step can be taken as simultaneously choosing both the column and the eluent of the solid-support chromatography [5]. Many different and effective CCC solvent systems have been proposed, studied and successfully employed and a number of different approaches have been established for selecting a suitable CCC solvent system [6-13]. Meanwhile, we must also admit that the elution mode is an important element that contributes to the success of the separation. It can improve the separation efficiency and save the separation time by using various elution modes. In recent years, studies on elution mode in CCC have made great strides, several novel elution modes have been developed and a number of related articles have been published. However, only a few reviews have presented this topic little [14, 15]. So we reviewed the progress of elution mode in counter-current chromatography and this review will give a brief summary of recently research progress on applications of different elution modes in CCC.

2. Advance in elution mode

Separation of complex sample is one of the development tendencies in CCC technique. In contrast with other chromatographic techniques such as liquid chromatography, electrophoresis, CCC has lower number of theoretical plates.

Consequently, it is inefficient in separation of complex samples. With conventional isocratic mode, it is a simple and effective method of isolating and purifying few major compounds from complex mixtures. However, it is difficulty in actual practice to separate more different compounds with a broad range of hydrophobicity. Fortunately, because CCC technique is an all-liquid method without solid phase, this means great flexibility in the choice of elution mode. Therefore, different elution modes have been developed and applied to deal with actual complex samples in CCC methodology.

2.1 Gradient elution

Gradient elution is very frequent practice for HPLC analysis and it can be accomplished through the changes in eluting medium or operation conditions. This method is increasingly used in CCC as well. Depending on the various form of change, there are usually of two kinds: stepwise gradient elution mode and linear gradient elution mode. In a stepwise elution mode, the elution condition is changed stepwise at one or several occasions. It can be treated as a composition of multiple steps of isocratic elution and several solutes may be eluted in each step. Stepwise elution mode is frequently used to separate complex samples in CCC. It generally includes the stepwise changes in mobile phase composition [16-18], flow rate [19, 20], pH value [21, 22] and salting-out concentration [23]. In a linear gradient elution mode, the elution condition is changed continuously toward condition is favourable for separation. During the whole gradient elution, the elution condition is sustained variation. It usually achieved through altering mobile phase composition [24] and pH value [25, 26]. In practice, the stepwise mode is more common method than linear mode and this might be because the gradient elution in liquid-liquid chromatography is quite different from liquid-solid chromatography and stepwise mode is relatively easy to implement.

In gradient elution of CCC, most the work involved change of mobile phase composition or flow rate. When the target solutes have a wide range of polarities and the conventional isocratic solvent system fails to provide an adequate separation of all target solutes, the most effective way of improving the separation is to change the mobile phase composition [27, 28]. With the rapid change in the polarity of the mobile phase, the solutes in the solvent system can be eluted faster. By the same token, the stepwise increase in flow rate was used in separation to save the time [29, 30]. The procedure is begun at low flaw rate, in course of time the solutes with small partition coefficient (*K*) values were eluted first. Then, increase the flow rate, which leads to faster elution of the remaining target solutes with higher *K* values. Recently, some gradient elution mode of pH value [31, 32] and salting-out concentration [23] was applied in CCC separation. By adjusting the pH value or salt concentration of the solvent system can improve the efficiency and achieve the best separation with a good manipulability and flexibility.

Gradient elution is a useful approach to perform separation for solutes with large difference in polarities. It has the advantages that significantly broadens the range of CCC application and considerably reduces the separation time. But its applications in CCC do not seem to be such easy matter and is not as straightforward as in HPLC. The major difficulty of gradient elution in CCC is any change of operation condition may induce the change of composition of stationary phase and loss of stationary phase retention (S_f), particularly in the change of mobile phase composition [18]. So, it is a requirement that the stationary phase in gradient elution remains relatively stable in the composition when the composition of mobile phase is rapidly changed during the separation. Not all liquid systems can be used to perform gradient elution in CCC experiments. It is generally assumed that during a gradient run the

change in stationary phase composition should be less than 20% to prevent instability of the stationary phase [33]. In practical applications, the ternary solvent systems like hexane/methanol/water, chloroform/methanol/water, hexane/1-butanol/water, ethyl acetate/1-butanol/water system and quaternary solvent systems like heptane or hexane/ethyl acetate/methanol/water have been proved to be useful and appropriate solvent systems for gradient elution due to their stability and a board range of polarity. Some studies suggested employing phase diagrams to build gradients and predict stability of the stationary phase and even calculate a composition of initial and final phases for gradient elution [34, 35]. Meanwhile, other beneficial attempt has been made. In literature [36], a 3 stepwise gradient elution combined with a descending stepwise flow rate gradient was introduced by Du's group. In this experiment, the lower phase of solvent system composed of n-hexane/1-butanol/0.05M NaOH (5/1/6, v/v) was used as the stationary phase and the upper phase was used as the initial mobile phase. To decrease the loss of stationary phase, the flow rates were significantly reduced from the initial 5.0 mL/min to 3.0 mL/min (step 1), 2.0 mL/min (step 2) and 1.5 mL/min (step 3) with the stepwise rise of 1-butanol content in the mobile phase from initial 5:1 to 1: 1 (step 1), 1: 2 (step 2) and 1: 4 (step 3) consisting of n-hexane/1-butanol, and the retention of stationary phase during the gradient steps decreased to 67%, to 65%, and to 64%, respectively. As is well known, reducing the flow rate helps to improve retention of stationary phase in CCC. In this example, the authors reduced the flow rate while changed the mobile phase to minimize the adverse effects on stationary phase and provide satisfactory separation for the target compounds. Four ursane triterpenoids (asiatic acid, madecassic acid, asiaticoside and

madecassoside) have been successfully separated by gradient elution method in a single-step CCC separation.

2.2 Dual-mode elution and multiple dual-mode elution

The dual-mode elution is a unique elution method in CCC. CCC instruments can run in either normal-phase or reverse-phase modes and freely switch between the both modes during the running. This process which uses both normal mode and reverse mode (in CPC, they also be called ascending mode and descending mode) to elute the solutes in the same separation is called dual-mode elution (see Fig. 1) [37-39]. In CCC, the dual-mode elution can be easily achieved by using a switching valve. It begins with classical elution, then changes stationary phase to mobile phase and simultaneously switches the circulation direction at a certain time during the separation process. The dual-mode elution can quickly elute the solutes with high K values that have strong affinity for the original stationary phase, which save the separation time and improve the separation efficiency. Meanwhile, both phases of the biphasic solvent system can be employed as mobile phase and as a result the solvent waste can be reduced.

In dual-mode elution, the separation progress was continued after the phase reversal. The K value of a solute will become $1/K$ after the elution mode was switched. So, the higher the retention of the solute before the phase reversal, the faster it was eluted after the phase reversal. Agnely and Thiébaut [40] extensively studied the retention and resolution in dual-mode elution in theory and compared to those of classical elution. They educed the equation for the computation of maximum retention volume (V_{max}) in dual-mode elution and pointed out the V_{max} is nothing to do with the K value of the solute. It only depends on the elution volume in classical elution and the composition of the column. In the special case in which the elution volume in classical elution

equals column volume, the V_{max} is equal to twice column volume. This means that regardless of the solvent system, phase ration in the column and *K* values of solutes, all solutes will be eluted in dual-mode elution by eluting a column volume of mobile phase in classical elution step and eluting a column volume of mobile phase (original stationary phase) after phase reversal. Their model also indicated that dual-mode elution can increase the resolution but it depends on some conditions such as solute, sample volume and solvent system. The study of Nazim Mekaoui and Alain Berthod [41] has also confirmed this claim. Their study demonstrated that the increase of resolution is relevant to the *K* value of solute in this solvent system. For low *K* value solute, the ration (Rs_{DM}/Rs_{CM}) of resolution factors obtained in dual-mode elution (Rs_{DM}) and classical elution (Rs_{CM}) is above 1. It means the dual-mode elution provide better resolution than the classical elution mode. But for high *K* value solute, the ration is always less than 1. It means dual-mode elution leads to a decrease of resolution for solute with high *K* value. However, considering the long separation time and large solvent consumption for separation of solute with high *K* value using classical elution, dual-mode elution has enormous potential for this situation and is a better alternative.

The multiple dual-mode elution is an extension of dual-mode elution and involves a series of consecutive dual-mode steps [42, 43] and it also be named intermittent dual CCC [44]. This approach can improve the resolution of target peaks and can be used to deal with solutes with close partition coefficients such as enantiomers [45, 46]. The solutes in the column eluted back and forth until the two peaks are separated through a series of switch between normal and reverse mode elution operation. In multiple dual-mode elution, the peak resolution increase depend on the number of repetitions of the multiple dual-mode elution steps (see Fig. 2). Yang et al [44] established a mathematical

model to predict retention time of the compounds and this model was validated by a series of basic studies on both hydrodynamic and hydrostatic CCC systems. They fully investigated the effects of dual cycle times and flow rate for each elution period on peak resolution. The results indicated that in both CCC systems, the resolution of solutes would increase with the switching times of the phase role because of the increased elution time. Meanwhile, they noted that the backward elution time interval and backward flow rate also have an influence on the resolution. In addition, Nazim Mekaoui and Alain Berthod [41] also theoretically analysed the influences of elution liquid phase volumes on the resolution and validated their theory through modelling the separation of two solutes. Their model showed good agreement with the experimental results in the prediction of elution and improved resolution. Meanwhile, they through that the total elution volume plays a more important role in resolution increase of multiple dual-mode elution than the number of switching steps. Recently, Kostanyan et al [47] developed and validated a model which can be used to select optimal process conditions for the CCC separation using multiple dual-mode elution through the mathematical description of the elution with sample loading and varying from cycle to cycle phase flow durations. It is demonstrated that proper selection of the duration of individual cycles can greatly increase the separation efficiency of CCC columns.

Here, we argue that though the multiple dual-mode elution was improved and innovated on the basis of dual-mode elution, it was developed to achieve different separation goals with dual-mode elution. The dual-mode elution is a very effective method for rapid separation of compounds with extremely different *K* values from a complex sample without sample loss, while multiple dual-mode elution is primarily suitable for separation of compounds with

238 extremely different or similar *K* values. In the former case, it can be seen as a
239 mere repetition of dual-mode elution separation. The solutes can be eluted
240 from the both ends of the column. The solutes with low *K* values eluted from
241 one end of column and the solutes with high *K* values eluted from the other
242 end. Then the sample can be re-injected and begin the next separation. In this
243 way, it meets a semi-continuous process with a classical sample injection and
244 which only requires a single column [42]. In a sense, this way achieved a
245 simulated intermittent counter-current extraction on the general CCC
246 instruments. In the latter case, the solutes can be separated after several
247 phase inversion cycles by keep them moving back and forth in the column and
248 without out from the column during the separation. At last, the solutes would
249 be eluted out from the same end of column.

250 2.3 Recycling elution

251 Recycling elution previously has been used in the preparative LC for
252 separating some solutes difficult to separate because this mode can improve
253 the resolution factor [48]. In CCC, this elution mode also has been employed to
254 separate some natural compounds [49-51], epimers [52] and enantiomers [53]
255 which have quite low resolution factor. The recycling elution mode can be
256 easily achieved by connecting the outlet of detector with inlet of mobile phase
257 pump through tube and a valve. This methodology will extend the separation
258 time, but the solvent consumption remains the same. These merits make
259 recycling elution mode an attractive alternative, but there is a prominent
260 contradiction, peak extension, that restricts its real application. During the
261 cycle, solute peaks become broader and broader with the increasing number
262 of cycles. When the overlap of peaks is observed, the cycle must be stopped
263 (see Fig. 3). So, it means this method is hardly fit for the simultaneous
264 separation of several solutes [52]. But we can find that this method is

particularly suited for some binary separation, such as chiral separation since it can remarkably improve the resolution factor for target enantiomers without extra consumption of chiral selector and solvent.

Both the multiple dual-mode elution and recycling elution mode are could improve the resolution of target solutes peaks by the technical methods extending the length of the CCC column. But they have their own distinguishing features and can be used to deal with different situation to achieve their respective goals. A comparison of multiple dual-mode elution and recycling elution mode was summarized in Table 1.

2.4 Extrusion elution

The extrusion elution in CCC was been developed to handle the complex samples which contain solutes with a large range of *K* values. Compared with other elution modes in CCC, it can extensively extend the hydrophobicity window and enhance the separation ability of a single biphasic liquid system. This elution mode mainly includes elution-extrusion elution [54, 55] and back-extrusion elution [56, 57].

The elution-extrusion elution involves two processes: traditional elution and stationary phase extrusion procedure. In this way those solutes which highly retained in the column can be rapidly eluted and the consumption of solvent and separation time can be considerably reduced. It is generally considered that a full elution-extrusion elution includes three steps: classical elution, sweeping elution and extrusion [58]. The first step is a traditional CCC elution. After eluting a certain volume of mobile phase, the elution liquid is changed from mobile phase to stationary phase and the elution is continued. This is the sweeping elution and the mobile phase in the column will be replaced by the stationary phase. The solutes with lower *K* values will be eluted with the mobile phase. In the third step, stationary phase is continued to pump into the

column and the solutes with high *K* values will be pushed out accompany with the stationary phase in the column according to their *K* values order. After this step, the column was filled again by stationary phase and can be prepared for equilibration to begin a next separation. The major advantage of elution-extrusion elution is that it makes the best use of the character of the liquid stationary phase in CCC. It can rapidly elute all solutes in the column without any irreversible adsorption and the most of them be separated with acceptable peak resolution. Theoretically it could extend the reachable polarity range from zero to infinity [58]. In fact, it did dramatically expand the interval of the polarity continuum and usually the solutes with *K* values lie between 0.25 and 16 can be separated with optimal resolution, whereas solutes out the range tend to elute near the void volume ($0 < K < 0.25$) or the end of the elution ($16 < K < \infty$) [59].

The elution-extrusion elution garnered favourable attention because it can quickly separate compounds with an extended polarity range. Berthod et al [54] [58] theoretically analysed and discussed the behaviour in elution-extrusion elution CCC and derived equations for retention volumes, peak widths, resolution factors and distribution constants. Those equations have proven can be used for a proper and accurate prediction of peak position and peak width in an elution-extrusion elution and calculate the distribution constants from the solutes retention volumes. They demonstrated that this approach can dramatically improve the efficiency of CCC and enable CCC technique to be a fast separation technique in the case of complex samples and high-throughput separation technique in rapid screening of numerous samples [60]. So, it has now come into more widespread use [61, 62].

In elution-extrusion elution, the target solutes are separated and their resolution factors increase with increasing elution volume and reach the

highest point during the sweeping elution step. In the third step, the solutes are simply pushed out with the stationary phase. So, the time point when the eluent is changed into the stationary phase and start sweeping elution, it usually expressed as switch volume (marked as V_{CM}) is an important operating parameter. The lower the value of V_{CM}, the need of eluting solvent and separation time is less and the peaks of solutes during the extrusion step are narrower. Meanwhile, the value of V_{CM} should be sufficiently high to assure complete separation of the solutes which retain in the column after the classical elution step. Kostanyan [63] has established an equilibrium cell model to describe the separation process in elution-extrusion elution and the model also indicated that the separation process is controlled by the value of V_{CM} and column efficiency. In the case of the selection of V_{CM}, the principle need to be emphasised: in selecting, under the precondition to satisfy the separation of the solutes which remain in the column after the eluent changed, the value of V_{CM} should be as low as possible in order to save the solvent and get narrow solutes band in the process of extrusion. Meanwhile, high column efficiency could reduce the V_{CM}. Furthermore, this study developed some equations which can help to select the appropriate value of V_{CM} for an optimal elution-extrusion elution separation.

Recently, a novel overlapping elution-extrusion elution CCC was developed as a variation of elution-extrusion elution CCC and applied to separation of compounds from natural complex extraction [64]. In this method, the sample was injected before the equilibrium was established throughout the column. The mobile phase was be pumped into the column to perform the equilibrium and during the same period the sample was been eluted. Moreover, all target solutes should be eluted during the classical elution and sweeping elution step. Then, if performing a repeated separation, the mobile phase will be introduced

346 into the column and the next sample was injected during the extrusion step
347 rather than to wait until the extrusion is finished. In this way, the separation
348 time and solvent consumption will be further saved. In the literature [64], the
349 author compared the standard and the overlapping elution-extrusion elution
350 CCC by repeated Isolation of andrographolides from the extract of plant. The
351 results indicated that the overlapping elution-extrusion elution can save about
352 40% solvent and 20% time in a single separation and can save more time in
353 repeated separation. Because the switch of liquid and injection were being
354 done before the equilibrium was established, they are two important
355 parameters should be carefully determined. The author also gave some rulers
356 for selecting these parameters.

357 The other extrusion elution mode has been developed is back-extrusion
358 elution [56, 57]. Similar with elution-extrusion elution, the first step in back-
359 extrusion elution also is classical elution. But in the next step, the liquid mobile
360 phase is maintained and the elution direction is changed, so the stationary
361 phase and the contained solutes will be extruded from another end of column.
362 Lu et al [56, 57] compared the performance of both elution-extrusion and
363 back-extrusion elution through separation for the extract of natural plant (see
364 Fig. 4). They found in the back-extrusion elution, some solutes that still
365 retained in the column after the classical elution step is finished will appear the
366 "echo" peaks in the extrusion step. This adverse effect can be mitigated by
367 raising the elution volume of V_{CM}, but it will increase the separation time and
368 solvent consumption. Meanwhile, they summarized the distinct features of
369 those two extrusion elution modes (see Table 2).

370 2.5 Cocurrent elution

The cocurrent elution, as its name implies, is an elution mode which both the mobile phase and stationary phase are moving in the column at the same direction. But the stationary move more slowly than mobile phase [65]. It can be considered as "true" moving bed chromatography because the both phases inside the column are moving and be treated as a continuous CCC [66].

Bethod and Hassoun fully studied and was summarized the cocurrent elution mode in CCC using a mixture sample of five steroid compounds (prednisone, prednisolone acetate, testosterone, estrone and cholesterol) with widely differing polarities ($0.12<K<40$) [67]. They tested and compared the chromatographic retention behaviour through the separation of the five steroid compounds at classical elution and concurrent elution with different flow rates of stationary phase. The results indicated that cocurrent elution can dramatically decrease the retention time and elution volumes of the solutes with high K values. We calculated the decrease in percentage of those figures using the date in literature [67] and the results were listed in Table 3. From the results, we can see that cocurrent elution not only substantially reduced the retention time and peak width, but also greatly increased the column plate number. However, the resolution factor decreased with the stationary phase moved. In addition, these trends become progressively obvious with increasing K value and flow rate of stationary phase. The retention of solute depends on the volume of the stationary phase in the column. But in concurrent elution, the stationary phase is also moving in the column as the same direction with mobile phase. So the solutes in the column are carried along with the stationary phase while they eluted by the mobile phase. And it can be considered that the distance which a solute traversed in such a movement is shorter than the column actually is. So the separation time and volume

consumption are decreased and the band broadening is suppressed. Meanwhile, the solution factor also decreased.

But there is a defect in the concurrent elution. It is the serious noise in the detection, which was caused by the two immiscible liquid phases simultaneously flow through the detector. Most classical chromatographic detectors cannot deal with this situation. Adding a clarifying agent post-column and using the mass spectrometer or the evaporative light scattering detector can partially solve this problem [67].

2.6 pH-zone-refining CCC

The pH-zone-refining CCC was developed upon the peak sharpening phenomenon caused by change of pH value [68]. It is a powerful preoperative scale elution mode that can remarkable enhance the sample loading capacity [69], and sometimes even can exceed 10 times [68]. This mode was primarily used in the separation and purification of compounds whose electric charge depends on the pH value, such as organic acids [70-72], alkaloids [73-75], amino acids [76], peptides [77, 78]. The compounds would be eluted with highly concentrated rectangular peaks according to their pKa values and hydrophobicity. This elution mode was widely used in separation of ionizable compounds since it was developed because of its notable advantages over conventional CCC (see Fig. 5). The origin, mechanism, application, procedures notice, advantages and limitations of pH-zone-refining CCC have been very fully described and discussed in the reviews by Yoichiro Ito and Ying Ma [79, 80]. Here is a brief description to the pH-zone refining CCC. In pH-zone-refining CCC, the acid/base was added in stationary phase as retainer and base/acid was added in mobile phase as eluter for acidic/basic compounds. Usually the organic acid, such as trifluoroacetic acid, that is used as retainer for the acidic analytes while the inorganic base is used as eluter, for example, NH_3, Na_2CO_3 or

NaOH. In the same way, the organic base, such as triethylamine, that is used as retainers for the basic analytes while the inorganic acid is used as eluter, such as HCl [80]. It must be noted that in pH-zone-refining CCC the eluting time of the solute is depending on the concentration ration of the retainer in the stationary phase and the eluter in the mobile phase. The most commonly-used way is equimolar concentration of retainer and eluter, typically 10-20 millimole each.

The pH-zone-refining CCC and pH gradient elution both are attributable to pH-related CCC techniques and rely on the variations in the solute retention behaviour caused by varying pH value to achieve the separation. However, there are some subtle and important differences. In pH-zone-refining CCC, it is usually to use almost equal molal concentration of acid/base or base/acid in stationary and mobile phase as retainer and eluter. The concentration of base or acid was added in the mobile phase as eluter is constant during the whole separation. But in pH gradient elution, most separation don't require acid/base retainer in stationary phase, and the concentration of base or acid in the mobile phase is increased continuously during the separation. The main differences between those two elution modes were summarized and listed in table 4. It should be pointed out that those two modes can be used in combination [32]. In this situation, it has characteristics of both of the two modes.

3. Summary

With the development of CCC technique, there are a variety of elution modes are available for CCC separation. Some of them are derived from other chromatographic technologies, such as gradient elution and recycling elution modes. And others are developed by fully utilizing its advantage of intrinsic flexibility which given by liquid nature of both the stationary and mobile phase

in CCC, such as dual-mode elution and multiple dual-mode elution, extrusion elution, cocurrent elution and pH-zone refining modes. By comparison with classical CCC elution mode, these elution modes have different characteristics and specific effects that make them desirable for certain applications. CCC user can select a suitable elution mode according to the real sample to meet different experimental requirements. A brief summary of the characteristic, recommended for target compound and principal merits in these elution modes were presented in Table 5. In addition, a combination of these elution modes can be used and sometimes is used for a better separation, such as the separation of main ganoderma triterpenoids using stepwise combined with pH-zone-refining CCC [81], separation of atropine and scopolamine using pH-zone-refining combined with counter-rotation and dual-mode elution [82], separation of nine compounds (caffeic acid, 6-hydroxyluteuolin-7-glucoside, 5,7,3′,4′-tetrahydroxy-6-methoxyflavanone-7-glucoside, nepitrin, rosmarinic acid, homoplantaginin, nepetin, hispidulin and 5,6,7,4′-tertrahydroxyflavone) using two-step CCC which elution-extrusion elution combined with classical elution and recycling elution [83]. The combination of the different elution modes can take the advantages of both elution modes, and show powerful separate capability and greatly improve the separation resolution. Therefore, provides a quick and easy way to obtain pure compounds from complex samples.

Meanwhile, there are some problems should be pay attention for employing these elution modes in separation real samples. The first is retention of stationary phase. In the CCC run procedure, a delicate equilibrium must be maintained between the mobile and stationary phases for a successful separation. Any subtle change of operating conditions will affect this equilibrium and result in loss of the stationary phase. Hence as far as the

choice of the solvent system is considered, the user should try to select it with higher hydrodynamic stability and obtain the highest possible stationary phase retention to minimize the possible loss of hydrodynamic equilibrium and overall separation efficiency which are suffered by the change of operating conditions. The second is detection problem. In some elution modes, the loss stationary phase result from change of operating conditions maybe continuously discharge with effluent from the column, which would lead to a dramatically increasing noise of elution curve. This can be prevented by using evaporative light scattering detection or mass spectrometer as well as using off-line detection, such as HPLC or TLC [7].

4. Conclusion

CCC is an effective and useful tool for separation and purification components from natural products, particularly in the preparative separation. The elution mode plays an essential role and fulfils important task in CCC separation. A right and appropriate elution mode would significantly improve the efficiency of CCC separation; dramatically extend the hydrophobicity ranges to the target compounds and greatly save the separation time and solvent. So, some different elution modes have been established and studied in the past decades. These elution modes provided more alternative choices for users to combat complex sample with CCC. The development and the application of those novel elution modes have grown notably, which made the CCC technique more flexible, greatly increased its usefulness and extended its applicable field. With the recent advances and novel developments in elution mode of CCC, it is evident that the great improvement in efficiency and selectivity has made predominant contribution in concentration and separation of active compounds from complex crude extraction. We hope this

review could help CCC user to better understand the technique and establish appropriate method for CCC separation.

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Figure 1. The results of Dual-mode separation and HPLC analysis. (A) Dual-mode separation of major components in P. tricuspidata. Experimental conditions: rotation speed: 1300 rpm; two-phase solvent system: ethyl acetate/acetonitrile/water (3/3/4, v/v); in descending mode (0–230 min), mobile phase: lower aqueous phase; in ascending mode (230–350 min), mobile phase: upper organic phase; flow rate: 2 mL/min; detection: 280 nm; sample: 500 mg P. tricuspidata crude extracts dissolved in 2 mL of a mixture of upper and lower phases. (B) HPLC chromatograms and UV spectra of crude extracts and isolated peak fractions (I–V). HPLC conditions: Hydrosphere C_{18} (250 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile and water; gradient elution, 0–5 min: 0% acetonitrile, 5–30 min: 0–30% acetonitrile, 30–40 min: 30–50% acetonitrile, 40.01–45 min: 100% acetonitrile; flow rate: 1 mL/min; sample injection volume, 10 μL; detection, 280 nm. Fraction peak (I): aromadendrin-3-O-β-D-glucopyranoside (1), fraction peak (II): *trans*-piceid (2), fraction peak (III): catechin (3), fraction peak (IV): resveratrol (4), fraction peak (V): engeletin (5) (Reproduced and adapted from Ref [38]).

Figure 2. Separations of N-(3, 5-dinitrobenzoyl)-(±)-leucine by chiral HSCCC with conventional and modified multiple dual-mode elution. (A) Schematic setup of multiple dual-mode elution operating system showing (a) Normal Phase elution mode (upper organic phase is the mobile phase) and (b) Reversed Phase elution mode (lower aqueous phase is the mobile phase). (B) Elution profiles obtained for the separation of N-(3, 5-dinitrobenzoyl)-(±)-leucine. Experimental conditions: solvent system: methyl t-butyl ether/50 mM phosphate buffer (pH 6.0) containing 90 mM (S)-naproxen in the upper phase (RP mode); flow rate: 1 mL/min; revolution: 2100 rpm; stationary phase retention: 84%. (a) One cycle; NP, 6min; (b) three cycles; NP, 6 min; time between cycles, 15 min; (c) modified multiple dual-mode elution (rotation is stopped during the NP period): one cycle; NP, 6min; (d) modified multiple dual-mode elution: three cycles, NP, 6 min; time between cycles, 15 min. One cycle corresponds to two phase inversions. (Reproduced and adapted from Ref [45]).

Figure 3. The results of HPLC analysis and recycling countercurrent chromatography separation. (A) HPLC analyses of mixed gambogic acid epimers on a C_8 column eluted with

787 CH_3CN/0.1% acetic acid/1, 4-dioxan (60/30/10, v/v). The flow rate was 1.0 mL/min, and the
788 effluents were monitored at 360 nm by a photodiode array detector. (B) Preparative
789 separation of gambogic acids by recycling countercurrent chromatography. Solvent system:
790 n-hexane/methanol/water (5/4/1, v/v); stationary phase: upper organic phase; mobile
791 phase: lower aqueous phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm; sample: 50
792 mg dissolved in 5mL of lower phase; peak I: gambogic acid; peak II: epigambogic acid
793 (Reproduced and adapted from Ref [52]).
794
795 Figure 4. Fractionation of an ethanol extract of *Piper longum* L. (A) HPLC analysis of the
796 crude extract. Column Zorbax XDB C_8 15 cm, mobile phase methanol/water (70/30, v/v) for
797 5 min, gradient from 70 to 90% 5–13 min, 90–95% 13–25 min, 1 mL/min. (B) Back-rxtrusion
798 with V_{CM} = 140 mL. (C) Elution-extrusion with V_{CM} = 140 mL. (D) Back-extrusion with V_{CM} =
799 350 mL. Liquid system: hexane/ethyl acetate/methanol/water (3/2/3/2, v/v), aqueous
800 mobile phase flow rate: 2.9 mL/min; machine volume: V_C = 140 mL; rotor rotation: 650 rpm;
801 V_M =93mL; V_S =47mL; S_f = 34%; UV detection: 254 nm. Sample injection: 50 mg of dry extract
802 dissolved in 1mL upper organic phase+1mL lower aqueous phase (Reproduced and adapted
803 from Ref [56]).
804
805 Figure 5. The results of pH-zone-refining CCC separation and HPLC analysis of alkaloids
806 extracted from G. elegans. (A) and (B) Chromatograms of pH-zone-refining CCC separation,
807 solvent system: MtBE/CH_3CN/water (3/1.5/4, v/v), 20 mM TEA in the upper organic
808 stationary phase and 10 mM HCl in the lower aqueous phase; sample size: 1.0 g (A) and 1.5
809 g (B); flow-rate: 2mL/min; detection: 254 nm; revolution speed: 850 rpm; retention of
810 stationary phase: 58.8% (A) and 58.3% (B). (C) HPLC and UV spectrometry analyses of crude
811 alkaloids and the purified fractions. Experimental conditions: Hypersil ODS_2 column (250
812 mm×4.6 mm I.D.); column temperature: 25 °C; mobile phase: methanol/0.05% butyl amine
813 in water (1/1, v/v); flow rate: 1.0 mL/min; detection: 256 nm; injection volume: 5 μL.
814 (Reproduced and adapted from Ref [75]).

815 Table 1. Comparison of the multiple dual-mode elution with recycling elution in CCC

	Multiple dual-mode elution	Recycling elution
Resolution	Increase with number of steps	Increase with number of cycles
Total separation time	Increased	Increased
Peak extension	Mild	Severe
Total solvent consumption	Increased	Constant
Target solutes	Could be used to separate multiple solutes	Could be suited for separation of analogues
Instrument modification	Needn't	Need to connect the outlet of detector and inlet of pump

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825 Table 2 Comparison of elution-extrusion with back-extrusion (The date were quoted from Ref [57]).

	Elution-extrusion elution mode	Back-extrusion elution mode
Advantage	Extensively enhance the polarity range of CCC separations	
	Extremely sharp peaks and satisfactory resolution factors	
	Extremely suitable for separation of complex samples	
	Save six times of separation duration, as well as 80% of liquid phase	
	Facility of sample pretreatment procedures that allowing the injection of most crude plant extracts	
	High-throughput separation	Simple operation
	Simple operation	
Drawback	Poor UV detection	Broad "echo" peaks
	Two pumps and a solvent selection valve to minimum the dead volume	Not continuous for high-throughput separation

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828 Table 3. Comparison of classical elution with concurrent elution under different flow rates of stationary phase (The date
829 were quoted from reference [67]).

Elution mode		Classical elution					Concurrent elution									
Stationary phase flow (F_S)		No flow					F_S=0.5 mL/min					F_S=1.5 mL/min				
compounds	K	V_R (mL)	t_R (min)	W_b (mL)	N plates	R_S	V_R (mL)	t_R (min)	W_b (mL)	N plates	R_S	V_R (mL)	t_R (min)	W_b (mL)	N plates	R_S
Prednisone	0.12	21.3	10.6	8.6	140		25.9 (21.60%)	10.3 (-2.83%)	7 (-18.60%)	220 (57.14%)		34.2 (60.56%)	9.8 (-7.55%)	11 (27.91%)	150 (6.67%)	
Prednisolone acetate	0.56	37.2	18.6	12	150	1.55	40.7 (9.41%)	16.3 (-12.37%)	12 (0)	180 (20%)	1.7(9.68%)	45.8 (23.12%)	13.1 (-29.57%)	11 (-8.33%)	270 (80%)	1.5 (-3.23%)
Testosterone	1.4	67	33.5	22.5	140	1.9	62.4 (-6.87%)	25 (-25.37%)	17 (-22.44%)	220 (57.14%)	1.6 (-15.79%)	57.5 (-14.18%)	16.4 (-51.04%)	11 (-51.11%)	440 (214.29%)	1.2 (-36.84%)
Estrone	4.6	183	91.5	60	150	2.8	106 (-42.08%)	42.5 (-53.55%)	27 (-55%)	250 (66.67%)	2.0 (-28.57%)	71.8 (-60.77%)	20.5 (-77.60%)	11 (-81.67%)	680 (353.33%)	1.2 (-57.14%)
Cholesterol	40	1460	730	460	160	6.2	166 (-88.63%)	66.2 (-90.93%)	36 (-92.17%)	340 (112.5%)	1.8 (-70.97%)	82.3 (-94.36%)	23.5 (-96.78%)	7 (-98.48%)	2200 (1275%)	0.7 (-88.71%)

830 Note: The percentages in the parentheses following the date are the ration of the change of concurrent elution to the classical elution. V_R: retention volume; t_R: retention
831 time; W_b: peak width at base; R_S: resolution factor
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835 Table 4. Comparison of the pH-zone-refining CCC with pH gradient elution.

	pH-zone-refining CCC	pH gradient elution
Retainer and eluter	Both retainer and eluter are necessary and the concentrations are constant during the separation	Eluter is necessary and the concentration even type are varying during the separation
Injection time	Before mobile phase was bumped	Before mobile phase was bumped or after hydrodynamic equilibrium was established depending on the situation whether start elution with neutral mobile phase
Chromatography peak	Often it is concentrated rectangular peak	Rarely it is rectangular peak
pH value range of eluent	Generally cover the neutral point	Generally do not contain neutral point (less than 7 when using acid eluter; more than 7 when using base eluter)

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839 Table 5. Brief summary of these elution modes

	Characteristics	Recommended for separation target	Principal merit
Gradient elution	Change flow rate, pH value, composition and salt concentration of mobile phase	Solutes in a proper range of *K* value	Reduce separation time and solvent consumption
Dual-mode elution	Change the phase role and circulation direction during the separation	Solutes in a very large range of *K* value	Reduce separation time and solvent consumption
Multiple dual-mode elution	Change the phase role and circulation direction several times during the separation	Solutes with extremely different or similar *K* values	Achieve semi-continuous process; improve resolution factor
Recycling elution	The mobile phase recycle in the column	Solutes with similar *K* values	Improve resolution factor without the increase consumption of solvent
Extrusion elution	Extrude stationary phase after classical elution step	Solutes in a very large range of *K* value	Reduce separation time and solvent consumption
Cocurrent elution	Both phases in the column are moving at the same direction with different speed	Solutes in a very large range of *K* value	Reduce separation time and solvent consumption
pH-zone refining	Use acid and base in both phases as retainer and eluter	Solutes whose electric charge depending on the pH value	Increase sample loading capacity; concentrate solutes in fractions;

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