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Overlapping elution–extrusion counter-current chromatography: A novel method for efficient purification of natural cytotoxic andrographolides from *Andrographis paniculata*

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ABSTRACT

Counter-current chromatography (CCC) is extremely useful for the separation, purification, and isolation of natural products. Recently, Berthod et al. [4,5] established an elution–extrusion CCC method in metabolic analysis by combining regular chromatographic elution with stationary-phase extrusion, which extends the hydrophobicity window of a counter-current separation. In this study, a novel overlapping elution–extrusion CCC method was developed and applied to the preparation of natural cytotoxic andrographolides from the aerial parts of *Andrographis paniculata*, a well-known Traditional Chinese Medicine (TCM) with potent anti-inflammatory effect and anti-cancer activity. Its theory was first developed, and then a series of CCC experiments were performed to investigate the efficiency of the method in the separation of the ethanol extracts from *A. paniculata*. Results show that overlapping elution–extrusion CCC is an efficient method to prepare a cytotoxic natural diterpenoid combination of 14-deoxy-andrographolide and 14-deoxy-11,12-didehydroandrographolide with the molar ratio of 1:2 as well as andrographolide using an optimized solvent system composed of hexane–ethyl acetate–ethanol–water (5:5:4:6, v/v) with an on-demand solvent preparation mode. All components obtained showed potent cytotoxic activity against human hepatocellular liver carcinoma cells HepG2 and doxorubicin-resistant R-HepG2 cells. Molecular structures have been identified by electrospray ionization mass spectrometry (ESI-MS), electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), one- and two-dimensional nuclear magnetic resonance (1D- and 2D-NMR). The method appears to be very useful for the high-throughput purification of natural products.

1. Introduction

Chromatography is a key technique to obtain pure bioactive natural or synthetic compounds for structural elucidation, pharmacological testing or development into therapeutics [1]. In the past few years several chromatographic techniques, such as thin-layer chromatography (TLC), column chromatography (CC), and high-performance liquid chromatography (HPLC) have been developed for resolving complex natural product extracts into pure components. Among chromatography techniques, counter-current chromatography (CCC) distinguishes itself by employing mobile and stationary phases that are both liquids [1]. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support matrix used in conventional chromatography. The method has been successfully applied to the analysis and separation of various natural products [2,3]. Historically, CCC is an effective but time-consuming technique requiring long run times (hours or even days) for separation. Recently, a series of new CCC techniques and apparatus have been introduced that increase the throughput of CCC. As a result, CCC is becoming a more high-performance, high-capacity, and high-throughput method for the isolation and purification of "small" molecules from complex natural samples [4–12].

The technique of elution–extrusion CCC [4,5] brings a new breakthrough for the CCC separation, which extends the "sweet spot" of high resolution in the CCC process and provides access to the otherwise practically unapproachable high-K portion of the high-resolution chromatograms in CCC. Its improved capacities of K targeting make elution–extrusion CCC a promising tool for the specific metabolomic fingerprinting and footprinting. Besides this,

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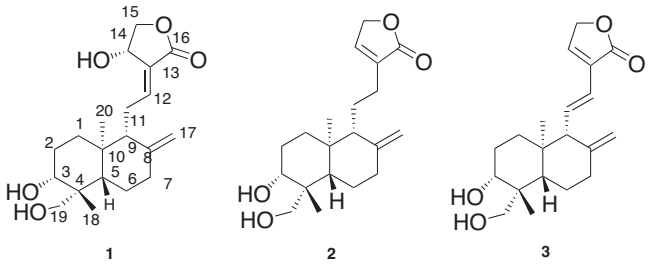

Fig. 1. Chemical structures of andrographolide (**1**), 14-deoxy-andrographolide (**2**), and 14-deoxy-11,12-didehydroandrographolide (**3**).

elution–extrusion CCC is an attractive method because the new stationary phase is recharged fully in the whole column of CCC once the elution–extrusion CCC experiment is finished [13]. Thus, the elution–extrusion CCC method seems suitable for the repeated preparation of target compounds.

The purpose of this work aims to establish the method "overlapping elution–extrusion CCC" for repeated preparation of natural products. Its basic theory principle was first analyzed, and then a series of experiments were performed. For an application, a famous Traditional Chinese Medicine, *Andrographis paniculata* was selected, which was widely used for centuries in China, India and other Southeastern Asian countries for the treatment of various diseases, such as respiratory infection, fever, bacterial dysentery and diarrhea [14]. Its diterpenoid constituents including andrographolide (**1**), 14-deoxy-andrographolide (**2**), and 14-deoxy-11,12-didehydroandrographolide (**3**) (Fig. 1) were reported to exhibit a wide spectrum of biological activities of therapeutic importance including antiviral [15], antimicrobial [16], anticancer [17], antioxidant [18] and anti-inflammatory [19] properties. Recent research indicated andrographolide can enhance 5-fluorouracil-induced apoptosis via the caspase-8-dependent mitochondrial pathway involving p53 participation in hepatocellular carcinoma (SMMC-7721) cells [20]. Biological activity testing requires the separation of pure components from traditional medicines in order to develop modern drugs. Thus far, a series of separation techniques, i.e. thin-layer chromatography (TLC), silica gel column chromatography, preparative high-performance liquid chromatography (HPLC) [21–23], and CCC have been developed to obtain pure diterpenoids such as andrographolide [20,24] and neoandrographolide [24] for further pharmacology experiments. However, there is no paper to report the repeated isolation of andrographolides from the extract of *A. paniculata*. To the best of our knowledge, this is a first application of overlapping elution–extrusion CCC for the repeated purification of andrographolides.

2. Theory

2.1. Elution–extrusion CCC method [4,5]

The elution–extrusion CCC method was initially proposed by Conway [25], who called it column extrusion. He proposed to use compressed nitrogen or air to extrude the whole CCC column content. He recommended backward extrusion for very highly retained solutes, still located at the column head, and forward extrusion for solutes closer to the column end [25]. The column extrusion method was used in several works [26–28] and fully developed by Berthod et al. [4,5] for the rapid screening of the analytes contained in a complex mixture.

Generally speaking, an elution–extrusion counter-current chromatography (EECCC) process comprises three stages: preparation, elution, and extrusion [5] as shown in Fig. 2.

The preparation stage establishes an equilibrium between both phases in the rotating column by filling first with the stationary phase before pumping the mobile phase until the hydrodynamic equilibrium has established. In the classical elution stage (I) the sample is injected and the solutes are eluted by the mobile phase. This mode is called "injection after equilibrium" (Fig. 2A). Injection may also be performed in the mode "injection before equilibrium" (Fig. 2B). In this case the column is entirely filled with stationary phase and eluted with the mobile phase follows injection. The classical elution may be ended at a predetermined volume (V_{CM}, also called the switch volume) of mobile phase. As described by the previous works [4,5], the solute retention volume V_{Ri}, is linked to their distribution ratios or partition coefficients, K_i, by the classical equation

$$V_{Ri} = V_M + K_i V_S \quad (1)$$

with V_M and V_S being the mobile and stationary phase volumes contained in the CCC column after the dynamic equilibrium. The total CCC column volume $V_C = V_M + V_S$.

After elution by V_{CM}, the extrusion process is started by simply changing the pumped liquid phase to the original stationary phase. According to the differences of outflow, the extrusion process undergoes two different stage: sweep elution (II) and extrusion (III). During sweep elution, the outcoming liquid is still mobile phase. Throughout sweep elution, the relationship of V_{Ri} and K_i is still the same as classical elution Eq. (1) although the solvent pumped into the column is now the original stationary phase [4,5]. In the following extrusion (III), the CCC column and effluent contain only stationary phase, and thus the solute retention volume, V_{EECCCi}, can be expressed as

$$V_{EECCCi} = V_{CM} + V_C - \frac{V_{CM}}{K_i} \quad (2)$$

2.2. Key parameters for repeated elution–extrusion CCC separations

Besides providing a higher resolution and a shorter separation time, the elution–extrusion CCC method has a characteristic advantage over other CCC methods in that the final stage of elution extrusion prepares the column for equilibration to begin the next CCC operation. With this in mind, repeated elution–extrusion CCC can be performed. Fig. 2A and B shows typical repeated elution–extrusion CCC operations with "injection after equilibrium," and "injection before equilibrium," respectively. In both cases, the injection of a new sample can occur before the previous sample has completed eluted. For practical operation, several important concerns have been addressed as follows.

2.2.1. Injection before equilibrium

As shown in Fig. 2A and B, the time and solvent consumption required for repeated elution–extrusion CCC may be reduced by proposing that injection before equilibrium be performed. Injection before equilibrium is a common practice among CCC practitioners. Simply put, the sample is injected after the mobile phase has passed the sample injection loop and before the column has been completely equilibrated. Fig. 2B shows that injection before equilibrium reduces the mobile phase volume required by the CCC separation by V_M.

2.2.2. When to start extruding the solutes using the stationary phase?

Although the $2V_C$ elution–extrusion method ($V_{CM} = V_C$) [5,13,29] has been proven to be efficient for complex metabolomic analysis, the determination of the optimal value of V_{CM} is still important for a practical preparation of target compounds because of different

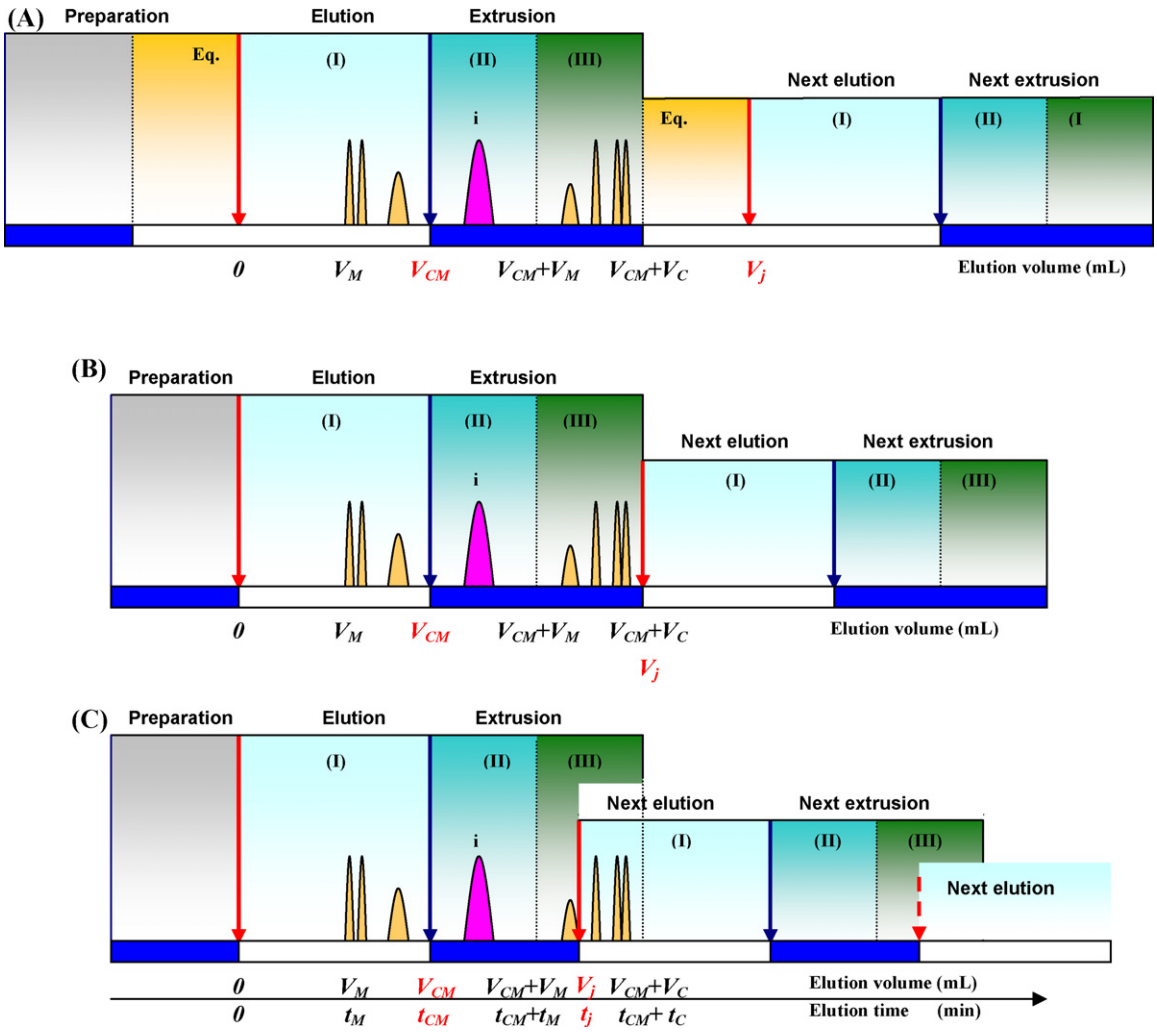

Fig. 2. Schematic representation of elution–extrusion CCC methods. (A) Injection after equilibrium; (B) injection before equilibrium; (C) sweep elution and extrusion at equilibrium. Eq., dynamic equilibrium of two phase. (I), elution; (II), sweep elution; (III), extrusion. Red arrow: the point to inject the sample and pump simultaneously mobile phase. Blue arrow: the point to switch pumped solvent from mobile phase to stationary phase. The white bar (below the each graph): pumping mobile phase into the CCC column. The blue bar: pumping stationary phase into the column. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

resolutions in different stages of the CCC process [5]. The resolution factor of a solute rises in the elution stage with the increase of elution volume and reaches the highest point during the sweep-elution. After this, the resolution factor of a solute diminishes in the later extrusion stage. Thus, it is essential to select an appropriate switch volume to start extruding the solutes in the column.

(1) During the sweep elution (II)

Theoretically, the effluents eluted during the sweep elution (II) (Fig. 2) should satisfy inequation (3).

$$V_{CM} \le V_{Ri} \le V_{CM} + V_M \quad (3)$$

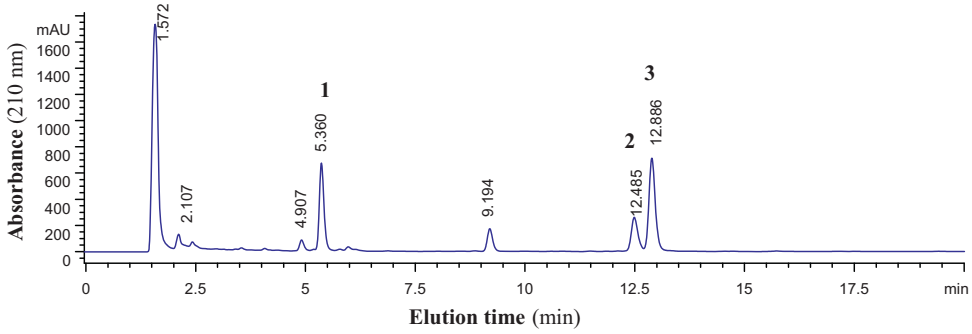

Fig. 3. HPLC analysis of the discolored ethanol extract of *A. paniculata*. (**1**) andrographolide; (**2**) 14-deoxy-andrographolide; (**3**) 14-deoxy-11,12-didehydroandrographolide. HPLC conditions: column: reversed-phase Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm I.D., 5 μm); mobile phase: acetonitrile–water (0–20 min, acetonitrile from to 30% to 50% and water from 70% to 50%; stop-time, 25 min.); flow rate: 0.8 mL/min; column temperature: 30 °C; UV detection, 210 nm.

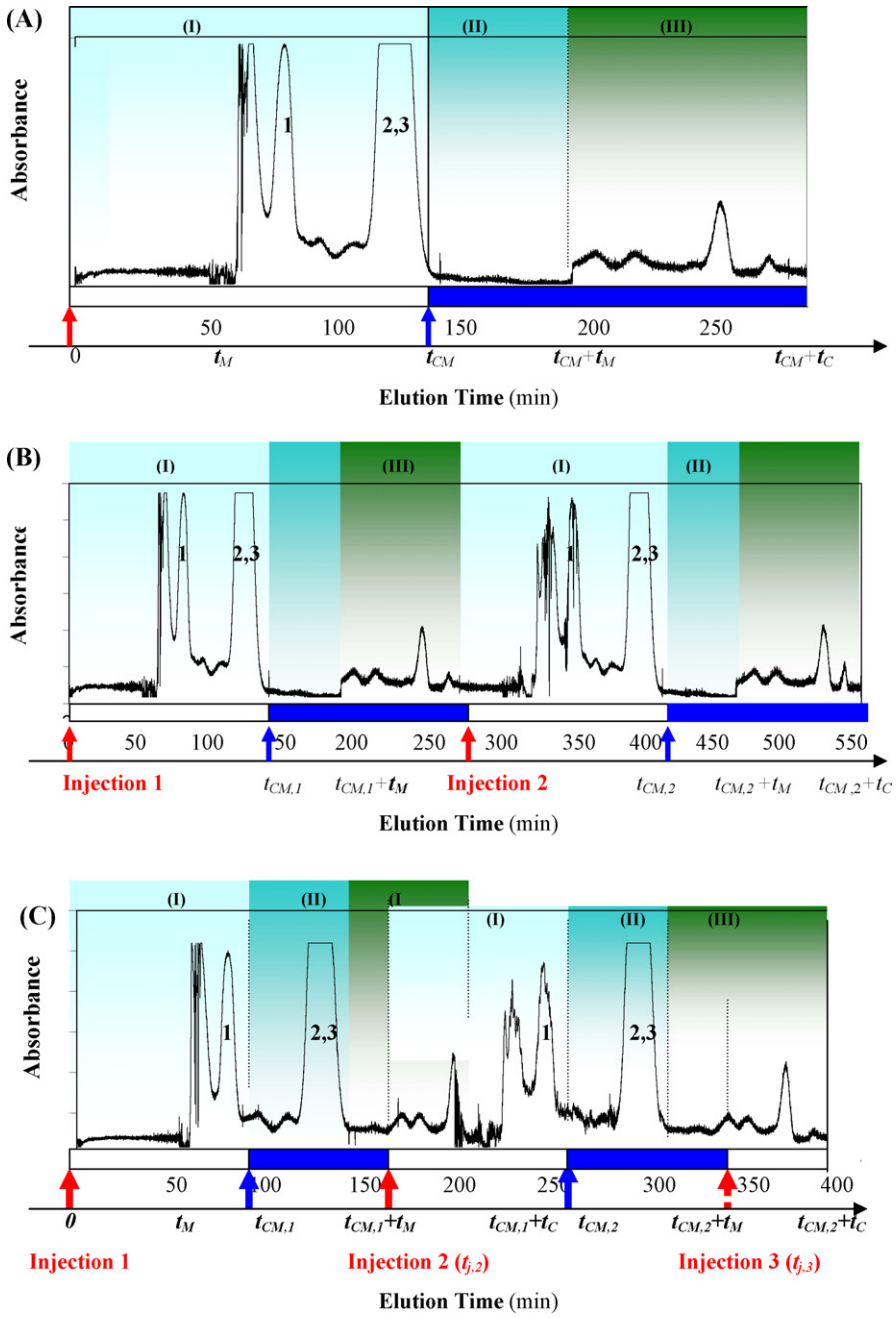

Fig. 4. The representative CCC profiles of the discolored ethanol extracts of *A. paniculata*. (A) Standard elution–extrusion CCC method (t_{CM} = 140 min) and (B) repeated elution–extrusion CCC ($t_{CM,1}$ = 140 min and $t_{CM,2}$ = 415 min, $t_{j,2}$ = 275 min); (C) the overlapping elution–extrusion CCC ($t_{CM,1}$ = 85 min and $t_{CM,2}$ = 250 min, $t_{j,2}$ = 165 min). Peak (**1**) corresponding to andrographolide (**1**) and peak (**2**,**3**) corresponding 14-deoxy-andrographolide (**2**) and 14-deoxy-11,12-didehydroandrographolide (**3**). Other conditions: injection mode: injection before equilibrium; elution mode: head-to-tail; flow rate: 2 mL/min; rotation speed: 850 rpm; column temperature: 30 °C; sample loading: 234.3 mg; UV detection: 254 nm; V_S = 160 mL and V_M = 110 mL; Solvent system 5:5:4:6 was prepared using an on-demand preparation mode [32], the components of upper phase and lower phase are illustrated in Table 2. (I), elution; (II), sweep elution; (III), extrusion. Red arrow: the point to inject the sample and pump simultaneously mobile phase. Blue arrow: the point to switch pumped solvent from mobile phase to stationary phase. Red dashed arrow: without third injection of sample. The white bar (below the each graph): the pumped solvent phase is lower phase used as mobile phase. The blue bar: the pumped solvent phase is upper phase used as stationary phase.

Thus, the switch volume should satisfy inequation (4).

$$V_{Ri} - V_M \le V_{CM} \le V_{Ri} \tag{4}$$

It should be noted that the peak width is not considered here. So, inequations (3) and (4) are only suitable for rapid determination of switch volume of the targets but not for all solutes of the target during the sweep elution. In practice, half of the solute is out of the sweep elution window when $V_{CM} = V_{Ri} - V_M$ or $V_{CM} = V_{Ri}$. Therefore, if all of a solute is required to elute in sweep elution, it should satisfy in equation (5).

$$V_{CM} + \frac{w_{bi}}{2} \le V_{Ri} \le V_{CM} + V_M - \frac{w_{bi}}{2} \tag{5}$$

In which W_{bi} is the peak width at base, $W_{bi} = 4V_{Ri}/\sqrt{N}$ (N, plate number). The value of V_{CM} is required to satisfy the equation

$$V_{Ri} + \frac{w_{bi}}{2} - V_M \le V_{CM} \le V_{Ri} - \frac{w_{bi}}{2} \tag{6}$$

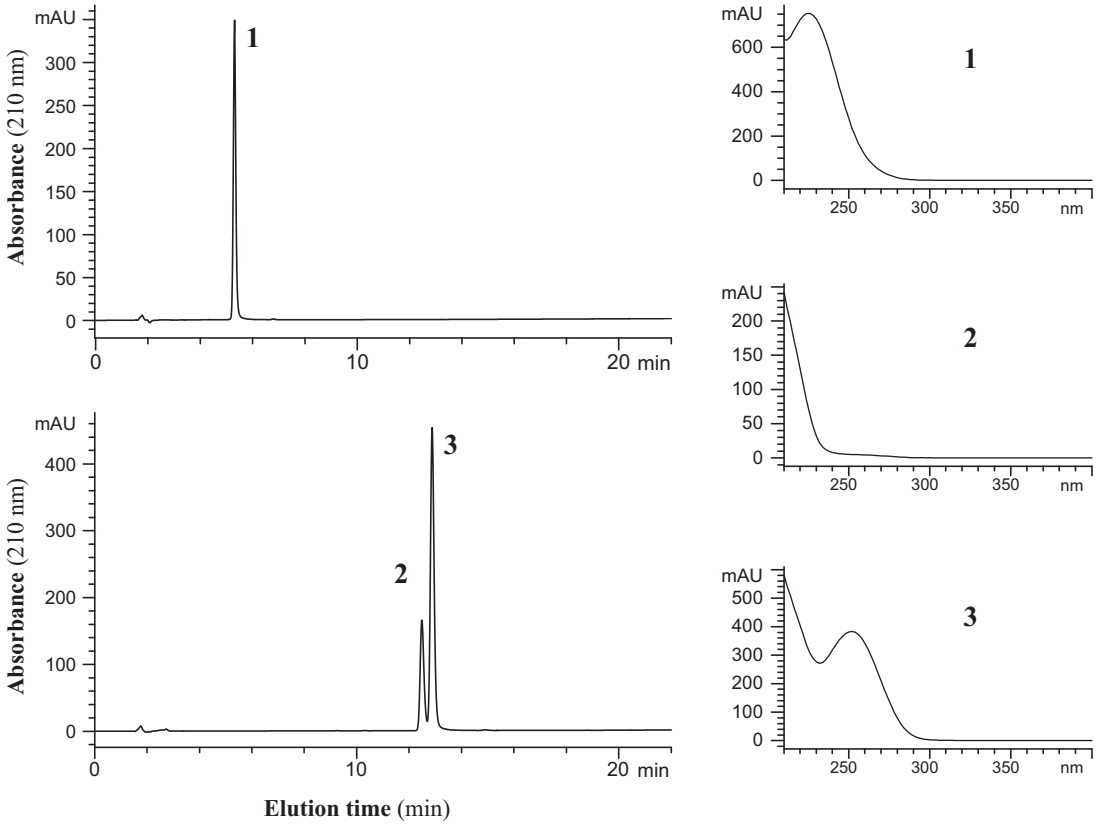

Fig. 5. Typical HPLC analysis of the products obtained by the overlapping elution–extrusion CCC.

In which retention volume (V_{Ri}) can be calculated by Eq. (1) from the shake flask experiment, the peak width and stationary phase retention volume (W_{bi}, and V_M) can be calculated from the known column efficiency (N, plate number) or previous experiences. Thus, once the solvent system for CCC is selected, the V_{CM} may be selected. There are two limitations of V_{CM} for the solutes during the sweep elution. One is the minimum value, $V_{CM,\min} = V_{Ri} + (w_{bi}/2) - V_M$, in which case, the required mobile phase for elution of the target component is the least and the saved mobile phase volume is equal to V_M, the volume of mobile phase contained in the CCC column after the dynamic equilibrium; the other is the maximum value, $V_{CM,\max} = V_{Ri} - (w_{bi}/2)$. Practically, the most solvent-saving method is to select the minimum value of the switch volume.

(2) During the extrusion (III)

The solute eluted in extrusion stage (III) should satisfy inequation (7).

$$V_{CM} + V_M \le V_{\mathrm{EECCC}i} \le V_{CM} + V_C \tag{7}$$

Thus the value of V_{CM} is required to satisfy inequation (8).

$$V_{CM} \le K_i V_S \tag{8}$$

Considering the effect of the peak width, the inequation becomes (9).

$$V_{CM} + V_M + \frac{w_{bi}}{2} \le V_{\mathrm{EECCC}i} \le V_{CM} + V_C - \frac{w_{bi}}{2} \tag{9}$$

However, the peak width of solute in the stage of extrusion changes with the change of V_{CM}, $W_{bi} = 4V_C\sqrt{V_{CM}/(NK_iV_S)}$ [5]. It has been shown that the peak width of the solute in the extrusion gets narrower [5]. Therefore, the usable selection range of the switch volume V_{CM} for eluting the solute in the stage (III), may be still determined by inequation (8).

In addition, several experiments indicate that the resolution of solutes in the extrusion stage is less than the resolutions in the stages of the elution and the sweep elution [4,5,30]. Therefore, it is optimal to select the sweep elution stage to elute the target solute.

2.2.3. When to inject the next sample?

The CCC operator does not have to wait until extrusion is finished to reintroduce the mobile phase into the column. In fact, the next dynamic equilibration process may be initiated after extrusion (III) has begun. In addition, injection before equilibrium may be applied at this point as well. In order to avoid overlapping K highly retained solutes from the first run with $K = 0$ solutes from the second run, the time point to inject the next sample must be adjusted according to the stationary phase retention volume ratio. Considering that the method "injection after equilibrium" requires an equilibrium process which is not time- and solvent-saving [31], here we only discuss the process using the method "injection before equilibrium" (Fig. 2B and C).

If the retention of stationary phase, S_F is more than 50%, namely, $S_F \ge 50\% \Leftrightarrow V_S \ge V_M$, the required mobile phase volume for the equilibrium being equal to V_M, is less than the volume of stationary phase V_S retained in the column. Usually, sample injection is done after the mobile phase has flowed past the sample loop. Thus, the next injection point V_j (the eluted volume when the next sample is injected) should satisfy inequation (10).

$$V_j \ge V_{CM} + V_C - V_M = V_{CM} + V_S \tag{10}$$

The earliest injection may start at $V_{j,\min} = V_{CM} + V_S$, in which case the saved mobile phase volume is equal to V_M.

Or else, if $S_F \leq 50\% \Leftrightarrow V_S \leq V_M$, then the required elution volume V_j should satisfy inequation (11).

$$V_j \geq V_{CM} + V_M \quad (11)$$

The earliest injection may be set at $V_{j,\min} = V_{CM} + V_M$, in which case the saved mobile phase is equal to V_S. Clearly, if injection is too early, the lead peaks of the new sample may overlap the previous sample constituents.

2.3. Overlapping extrusion and equilibrium

The preceding theoretical analyses showed that there are three ways to reduce separation time and solvent consumption. Taking into account the resolution and separation efficiency, an overlapping elution–extrusion CCC method is proposed. Fig. 2C shows a representative example of the method. The targeted compound (i) is eluted in the sweep elution (II) while the following injection of sample is done during the extrusion (III). The value of V_{CM} is determined according to inequation (6). The inequation (4) is also used to estimate V_{CM} without consideration of peak width. After sweep elution, the time to inject the next sample is selected according to inequations (10) or (11) depending on the stationary phase retention.

It should be noted that the above theoretical analyses are based on the volume model of the solute retention, which can be transformed into the alternative time model of the solute retention by a simple mathematic calculation

$$V = F \times t \quad (12)$$

where V is volume (mL), F is flow rate (mL/min), and t is time (min). Several parameters based on the time model have been illustrated in Fig. 2C.

3. Experimental

3.1. Apparatus

The CCC instrument employed in the present study was a TBE-300A high-speed CCC (Tauto Biotech. Co., Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (i.d. of the tubing, 1.8 mm; total column volume, 260 mL, and extra volume, 10 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β values of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus was regulated with a speed controller in the range between 0 and 1000 rpm. A TC 1050 constant-temperature circulating bath (Tauto Biotech. Co., Ltd., Shanghai, China) was used to control the temperature. In addition, the CCC system was equipped with a P270 metering pump, a UV 230$^+$ spectrometer (Elite Analytical Instrument Co., Ltd., Dalian, China), a BSZ-100 fraction collector and an EC2000 ChemStation (Elite Analytical Instrument Co., Ltd., Dalian, China).

An Agilent 1100 system HPLC was used for the analysis of the extract and a number of fractions. It was equipped with a G1379A degasser, a G1311A QuatPump, a G1367A well-plate (thermostatted) autosampler (Wpals), a G1316A column oven, a G1315B diode assay detector (DAD), and an Agilent ChemStation for LC.

3.2. Reagents

All organic solvents used for CCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. The water was purified by means of a water purifier (18.2 MΩ) (Wanjie Water Treatment Equipment Co., Ltd., Hangzhou, China) and used for the preparation of all solutions and the dilutions. The solvents used for the HPLC or GC analysis were of chromatographic grade. Acetonitrile and methanol were purchased from Merck, Darmstadt, Germany. Hexane, ethyl acetate and tetrahydrofuran were purchased from Siyou Biology Medical-Tech Co., Ltd., Tianjin, China. Ethanol was purchased from Tedia, USA.

The aerial part of *A. paniculata* was purchased from Huqingyutang Museum of Traditional Chinese Medicines (Hangzhou, China), and was identified by the Institute of Plant Science, College of Life Science, Zhejiang University, China, and its voucher specimen number is P06-1072.

3.3. Preparation of the discolored ethanol extract

The aerial parts of *A. paniculata* (101.07 g) were powdered and extracted three times at the room temperature with 95% ethanol (2000 mL each time). Then, the extracts were combined and filtered. The filtrate was concentrated to one third of the original volume by rotary vaporization at 46 °C under reduced pressure. The residue was re-extracted in a hot reflux device with 10 g activated charcoal. Then, the extract was filtered and the filtrate was evaporated under reduced pressure, yielding 2.61 g of the decolored extract used as sample for HPLC analysis and following CCC separation.

3.4. Measurement of partition coefficient (K)

The solvent selection process required knowledge of partition coefficients at various solvent systems. The K values for the components were determined by HPLC analysis as follows: a small amount (1 mg) of the discolored ethanol extract was dissolved into equal volumes (800 μL) of aqueous phase (lower phase) and organic phase (upper phase) of the thoroughly equilibrated two-phase solvent system in a 2 mL test tube. After the equilibration was established, both the upper phase and the lower phase were directly analyzed by HPLC. The peak area of each compound in the upper phase and in the lower phase were recorded as A_1 and A_2, respectively. The K value was then calculated by the following equation: $K = A_1/A_2$.

3.5. On-demand preparation of two phase solvent and sample solution

The two phase solvent system was prepared using an on-demand solvent preparation mode without pre-saturation of the two-phase solvent [32]. The components of each phase of the selected solvent system were first analyzed by GC-FID [32]. Because water does not make any response to FID detector, the water volume fraction (V_4) in the phases was calculated by equation V_4=1 − V_1 − V_2 − V_3, where V_1, V_2, and V_3 are the volume fractions of hexane, ethyl acetate and ethanol, which were determined by GC-FID. Then each individual solvent was added into the solvent bottles to compose the solutions of upper phase and lower phase.

The sample solutions were prepared by dissolving the extract in a solvent mixture consisting of equal volumes of both upper and lower phases at a suitable concentration according to the preparative scale of CCC separation.

3.6. Preparative CCC separation procedure

3.6.1. Elution–extrusion CCC separation

Elution–extrusion CCC separation with the mode of injection before equilibrium was performed as follows: (1) the CCC column was first filled with upper phase of the solvent system as the stationary phase. (2) The apparatus was rotated at the desired rotation speed and the mobile phase was pumped into the CCC column from head to tail with a 2 mL/min flow rate. (3) When the mobile phase had passed the sample loop, the sample solution was injected. (4)

Table 1
The *K* values of andrographolide and its analogs in several solvent systems.

Solvent system	*K*[a]		
n-Hexane–ethyl acetate–ethanol–water (HEEtWat, v/v)	**1**[b]	**2**	**3**
7:3:6:4	0	0.04	0.036
6:4:5:5	0.034	0.181	0.178
5:5:4:6	0.306	1.023	1.018
4:6:3:7	1.218	6.612	6.898

[a] *K* value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.
[b] **1**, andrograpolide; **2**, 14-deoxy-andrographolide; **3**, 14-deoxy-11,12-didehydroandrographolide.

Once the elution volume reached the predetermined value of V_{CM}, the upper phase was pumped into the column with a 2 mL/min flow rate. The effluent was monitored at 254 nm and collected in 20 mL test tubes every 5 minutes. Collected fractions were combined according to the elution profile and analytical HPLC analysis.

3.6.2. Overlapping elution–extrusion CCC

After the all lower phase retained in the column was eluted, the CCC entered the extrusion process (III). During extrusion, the lower phase was reintroduced as the mobile phase and a new sample was injected through the injection valve once the elution volume reached the required elution volume V_j which was determined by inequation (10) or (11). The flow rate was kept at 2 mL/min.

3.7. HPLC analysis and identification of CCC fractions

The extracts and CCC peak fractions were analyzed by HPLC. The column used was a reversed-phase C18 column (Zorbax eclipse XDB-C18, 150 mm × 4.6 mm I.D., 5 μm) with a guard column (10 mm × 4.6 mm I.D., 5 μm). An acetonitrile–water system was used as the mobile phase in gradient mode as follows: 0–20 min, acetonitrile from 30% to 50% and water from 70% to 50%; stop-time, 25 min. The flow-rate of the mobile phase was 0.8 mL/min and the effluents were monitored at 210 nm by a DAD detector. Other conditions included: column temperature, 30 °C; and injection volume, 10 μL.

Identification of the CCC fractions was carried out by electrospray ionization mass spectrometry (ESI-MS), electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), one- and two-dimensional NMR spectra (1D- and 2D-NMR) including ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) 135, ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and hydrogen/deuterium exchange (also called H/D exchange) by D_2O. Positive ESI-MS analyses were performed using a Thermo Finnigan LCQ Deca XP Electrospray-Ion Trap-Mass Spectrometer. Positive high-resolution ESI-TOF-MS analyses were employed an Agilent 6210 Series LC/Time-of-Flight Mass spectrometers. 1D- and 2D-NMR experiments were carried out using a Bruker Advanced DMX 500 NMR spectrometer with dimethyl sulfoxide (DMSO) as solvent and tetramethylsilane (TMS) as an internal standard.

4. Results and discussion

4.1. HPLC analysis of the discolored ethanol extract

The discolored ethanol extract of *A. paniculata* was first analyzed by HPLC. As well known, the activated charcoal is a strong adsorptive material and is also widely used for solid-extract, adsorption, and discoloration of natural and synthetic complex chemicals. It is also known that the activated charcoal can adsorb the large amount of non-andrographolides material in the ethanol extracts such as green chlorophylls components but make a minor adsorptive loss of anrdographolides, the major pharmacologically active components of *A. paniculata* [22,33,34]. Therefore, using the activated charcoal to discolor the ethanol extract of *A. paniculata* has been accepted as a standard extract protocol in a number of schoolbooks in China [35]. In this work, we also used this protocol to obtain the extract of androgrpaholides under the recommended conditions as described in Section 3.3.

As expected, the activated charcoal removed some natural green coloring components without adsorptive loss of target andrographolides (Fig. 3). In the present analysis, three diterpenoids andrographolide (**1**), 14-deoxy-andrographolide (**2**), and 14-deoxy-11,12-didehydroandrographolide (**3**) were resolved by a reversed-phase Zorbax Eclipse XDB-C18 column with an acetonitrile–water gradient. However, in other HPLC analyses employing columns such as Zorbax Rx-C8 with a methanol–water system, the components **2** and **3** could not be separated efficiently as single peaks but showed an overlapped peak due to their similarity of chemical structure (Fig. 1) and physical properties.

4.2. Selection of the optimum two-phase system and solvent phase component analysis

Successful separation by CCC largely depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficients for targeted compounds (*K* from 0.25 to 8 and even to 16) [36]. A previous study [20] shows that andrographolide (**1**) can be prepared at high purity by use of the system composed of hexane–ethyl acetate–ethanol–water (HEEtWat). As shown in Table 1, the solvent system composed of hexane–ethyl acetate–ethanol–water with a volume ratio of 5:5:4:6 was found to be most suitable for CCC separation of these compounds. Its two phase components were analyzed using the reported GC method [32] as shown in Table 2. It must be pointed out that 14-deoxy-andrographolide (**2**), and 14-deoxy-11,12-didehydroandrographolide (**3**) have same partition coefficients in these HEEtWat systems (Table 1), thus they could not be completely separated using the current CCC solvent systems.

Table 2
The volume fraction composition for the solvent system of hexane–ethyl acetate–ethanol–water (5:5:4:6, v/v).

Phase	Composition (%, v/v)[a]			
	Hexane	Ethyl acetate	Ethanol	Water[b]
Upper phase	51.77 ± 1.59	39.23 ± 1.18	8.22 ± 0.14	0.78 ± 0.61
Lower phase	0.44 ± 0.06	12.63 ± 0.11	31.58 ± 1.07	55.35 ± 1.10

[a] The volume fractions of the hexane (V_1), ethyl acetate (V_2) and ethanol (V_3) in each upper phase and lower phase have been determined simultaneously by GC-FID using tetrahydrofuran as internal standard according to the reported process [32].
[b] The water volume fraction (V_4) in both phases was calculated by equation: $V_4 = 1 - V_1 - V_2 - V_3$. Therefore, the errors values for the water were obtained by multiple repeated injections. Clearly, the accuracy of the value for water will lower than one of other components of hexane, ethyl acetate and ethanol.

4.3. Typical CCC separation with the on-demand solvent preparation mode

For efficient saving of the solvent and time, an on-demand solvent preparation mode developed recently [29,32,37] was used. Based on the GC analysis data of Table 2, hexane, ethyl acetate, ethanol and water with the predetermined volume were added into the solvent bottles to constitute the upper and lower phase. After pumping full the stationary phase into the CCC column, the successful CCC separation was performed and the discolored ethanol extract of *A. paniculata* was separated. The volume of the stationary phase V_S = 160 mL and the volume of the mobile phase V_M = 110 mL, correspond to t_S = 80 min and t_M = 55 min. A switch time (t_{CM}) of 140 min was chosen (being 280 mL@2 mL/min flow rate) in order to accommodate the elution of all three target analytes during the classical elution stage.

4.4. Standard $2V_C$ methods for single and repeated elution–extrusion CCC

As show in Fig. 4A, peak (**1**) and (**2**,**3**) elute in the stage (I) of elution. Almost no peaks were found in the sweep elution (II) while there are some components in the extrusion (III).

After the first elution–extrusion CCC run, the new upper phase as the stationary phase was recharged fully in the CCC column, thus the next sample can be injected and the elution–extrusion CCC can be repeatedly performed. Fig. 4B shows a typical elution–extrusion CCC for two repeated preparation of andrographolides from *A. paniculata*. Clearly, there are no significant differences between the two repeated CCC separations in the standard $2V_C$ methods for elution–extrusion CCC. From each 234 mg sample, 9.3 mg andrographolide (**1**) with 95.2% purity, and 16.5 mg combination of 14-deoxy-andrographolide (**2**) and 14-deoxy-11,12-didehydroandrographolide (**3**) (molar ratio, 1:2) with 98.1% purity was obtained.

4.5. Overlapping elution–extrusion CCC combining the use of sweep elution and extrusion at equilibrium

According above theoretical proposal, the most highly retained target compound (**1**) and (**2**,**3**) should elute in the sweep elution. In view of the fact that the solute in the sweep elution has a higher resolution factor [5], here we only discuss a case where the peak (**1**) is still eluted in the classical elution (I) and the peak (**2**,**3**) is eluted in of the sweep elution (II).

4.5.1. Determination of the switch time

The switch time t_{CM} is a very important parameter. Using the time model of solute retention, the inequation (6) can be transformed as follows

$$t_{Ri} + \frac{w_{bi}}{2} - t_M \le t_{CM} \le t_{Ri} - \frac{w_{bi}}{2} \quad (13)$$

Thus, the suitable switch time for the sweep elution of the peak (**2**,**3**) is within 80 min ≤ t_{CM} ≤ 105 min with the 2 mL/min flow rate. Taking account of an efficient saving of the solvent phases and a more convenient operation, the optimum switch time t_{CM} for the separation of andrographolides was set at 85 min.

4.5.2. Determination of the next injection time for the sample

The next injection time t_j is another important parameter for an efficient saving of the next separation time and of solvent. The single or repeated elution–extrusion CCC runs showed an high stationary phase retention with $S_F \ge 50\% \Leftrightarrow V_S \ge V_M$, thus the inequation (10) was used to determine the time-range for injection

Table 3
Comparison of two elution–elution CCC methods for repeated purification of andrographolides.[a]

Elution–extrusion CCC	Injection no.	Injection time (t_j, min)	Switch time (t_{CM}, min)	Stages (min)[b,c]				Used solvent (mL)		Total duration (min)	Total time for 2 cycles (min)
				Equilibrium & elution	Elution	Sweep elution	Extrusion	Extrusion at equilibrium	Lower phase	Upper phase	Total		
Standard	Preparation								0	270			
	1	0	140	55[d]	85	55	80		280	270	550	275	
				(0–55)[e]	(55–140)	(140–195)	(195–275)					(0–275)	
	2	275	415	55	85	55	80		280	270	550	275	
				(275–330)	(330–415)	(415–470)	(470–550)					(275–550)	550
Overlapping	Preparation								0	270			
	1	0	85	55	30	55	25	55	170	160	330	220	
				(0–55)	(55–85)	(85–140)	(140–165)	(165–220)				(0–220)	
	2	165	250		30	55	25	55	170	160	330	220	
					(220–250)	(250–305)	(305–330)	(330–385)				(165–385)	385

[a] Upper phase was used as stationary phase, and lower phase as mobile phase.
[b] The lower phase as mobile phase has been pumped into the column during the stages of equilibrium & elution, elution, and extrusion at equilibrium.
[c] The upper phase as the stationary phase has been pumped into the column during the stages of sweep elution and extrusion.
[d] Duration.
[e] The interval (start time–end time).

Table 4
^{1}H and ^{13}C NMR chemical shifts (δ) of purified diterpenoids (DMSO).[a]

No.[b]	Andrographolide (1)		14-Deoxy-andrographolide (2)		14-Deoxy-11,12-didehydroandrographolide (3)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	1.20 (1H, o),[c] 1.70 (1H, o)	37.0	1.19 (1H, o), 1.73 (1H, o)	37.0	1.19 (1H, o), 1.33 (1H, o)	38.5
2	1.62 (1H, o), 1.68 (1H, o)	28.4	1.63 (2H, o)	28.4	1.63 (2H, o)	28.2
3	3.27 (1H, o)	79.0	3.22 (1H, o)	79.0	3.22 (1H, o)	79.2
4		42.8		42.8		42.9
5	1.20 (1H, o)	54.9	1.63 (1H, o)	55.1	1.19 (1H, o)	54.2
6	1.35 (1H, m), 1.74 (1H, o)	24.5	1.40 (1H, m), 1.73 (1H, o)	24.4	1.33 (1H, o), 1.73 (1H, o)	23.7
7	1.93 (1H, m), 2.32 (1H, m)	38.0	1.98 (1H, o), 2.36 (1H, o)	38.4	1.98 (1H, o), 2.36 (1H, o)	36.8
8		148.1		148.0		149.5
9	1.86 (1H, m)	56.0	1.19 (1H, o)	55.9	2.36 (1H, o)	61.1
10		39.1		39.3		38.8
11	2.50 (2H, m)	24.5	1.63 (2H, o)	22.1	6.75 (1H, dd, J = 10.1, 15.8)	134.8
12	6.62 (1H, dt, J = 6.7,1.7)	146.9	1.98 (1H, o), 2.36 (1H, o)	24.7	6.12 (1H, d, J = 15.8)	121.8
13		129.5		132.7		127.6
14	4.91 (1H, brs)	65.0	7.47 (1H, s)	147.5	7.66 (1H, s)	147.4
15	4.04 (1H, dd, J = 9.9,1.7), 4.40 (1H, dd, J = 9.9,6.0)	74.9	4.82 (2H, brs)	71.0	4.89 (2H, brs)	70.7
16		170.5		174.7		173.0
17	4.63 (1H, s), 4.82 (1H, s)	108.8	4.59 (1H, s), 4.83 (1H, s)	107.2	4.42 (1H, s), 4.73 (1H, s)	108.6
18	1.08 (3H, s)	23.6	1.07 (3H, s)	23.6	1.09 (3H, s)	23.6
19	3.25 (1H, o), 3.84 (1H, d, J = 10.8)	63.2	3.27 (1H, o), 3.83 (1H, d, J = 10.3)	63.2	3.27 (1H, o), 3.83 (1H, d, J = 10.3)	63.2
20	0.66 (3H, s)	15.3	0.60 (3H, s)	15.4	0.76 (3H, s)	16.0

[a] All spectra were recorded on a Bruker Advanced DMX 500 NMR spectrometer, in DMSO.
[b] The Carbon and proton signals were assigned unambiguously on ^{1}H and ^{13}C NMR, DEPT 135, ^{1}H–^{1}H COSY, HMQC and H/D exchange by D_2O.
[c] o, overlapped signals.

of the sample. The inequation (10) could be transformed into the time mode

$$t_j \geq t_{CM} + t_S \quad (14)$$

where $t_S = 80$ min, $t_{CM} = 85$ min, thus the next injection time will be $t_j \geq 165$ min with the 2 mL/min flow rate. A minimum extremum was selected for the next injection of sample.

4.5.3. Representative separation

A representative separation has been performed by using of the techniques that allow for the minimum amount of time and solvent. As shown in Fig. 4C, peaks (**1**) and (**2**,**3**) were well resolved with peak (**1**) being eluted in the classical elution (I), and peak (**2**,**3**) was eluted in the sweep elution (II). As a result (Fig. 5), 9.5 mg andrographolide (**1**) with 97.8% purity, and 17.8 mg combination of 14-deoxy-andrographolide (**2**) and 14-deoxy-11,12-didehydroandrographolide (**3**) (molar ratio, 1:2) with 98.5% purity, were obtained from the 234 mg of injection sample.

4.6. Comparison of two elution–extrusion CCC methods

Table 3 shows the results of two elution–extrusion CCC methods for repeated purification of andrographolides. Clearly, the overlapping elution–extrusion saves more time and solvent. The standard repeated elution–extrusion CCC method needs about 550 mL of biphasic solvent system including 280 mL lower phase and 270 mL upper phase besides the pre-pumped 270 mL stationary phase and for a duration of 275 min for each cycle. Whereas the overlapping elution–extrusion CCC needs only 330 mL of biphasic solvents including 170 mL lower phase and 160 mL upper phase and a

Table 5
ESI-MS analyses of purifed components by CCC.

Components	Molecular formula	ESI-TOF-MS					ESI-MS	
		Mass	Formula	Calculated Mass	Difference (ppm)	Ion	Mass	Ion
Andrographolide (**1**)	$C_{20}H_{30}O_5$	373.1985	$C_{20}H_{30}NaO_5$	373.19909	1.59	$[M+Na]^+$	373	$[M+Na]^+$
							723	$[2M+Na]^+$
14-Deoxy-andrographolide (**2**)	$C_{20}H_{30}O_4$	357.2036	$C_{20}H_{30}NaO_4$	357.20418	1.62	$[M+Na]^+$	357	$[M+Na]^+$
							689	$[2M+H]^+$
14-Deoxy-11,12-didehydroandrographolide (**3**)	$C_{20}H_{28}O_4$	355.1884	$C_{20}H_{28}NaO_4$	355.18853	0.36	$[M+Na]^+$	355	$[M+Na]^+$
							687	$[2M+Na]^+$

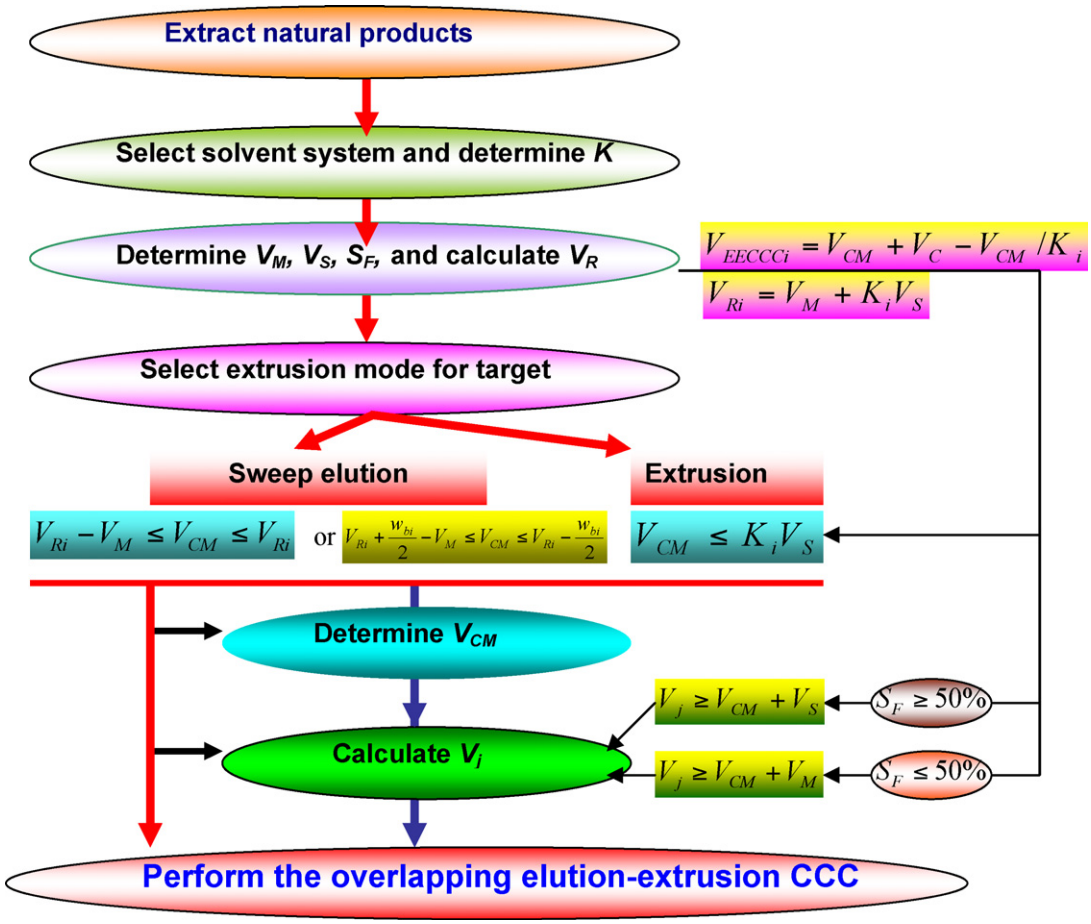

Fig. 6. A proposed protocol for the overlapping elution–extrusion CCC preparation of natural product targets.

duration of only 165 min per cycle. The representative separation (Fig. 4C) indicated that the overlapping elution–extrusion CCC technique saved 220 ml (equal to $2V_M$) mobile phase and 110 min (equal to $2t_M$) by comparison with repeated elution–extrusion CCC demonstrated in Fig. 4B.

It should be pointed that although HPLC analysis (Fig. 3) presented about only 7 major peaks, the discolored ethanol extracted from aerial part of *A. paniculata* was still relative complex because several peaks were not efficiently resolved except for peaks **1**, **2** and **3**. For example, the peak (retention time 1.57 min) maybe contained several materials because of its little retention under current HPLC conditions. In addition, as shown in Fig. 5, andrographolides **1**–**3** had different UV absorption spectra, thus these components could not be quantified by simple linear estimation of peak areas. Thus, the purity by HPLC analyses were validated by NMR. Andrographolides (**1**) (9.5 mg, 97.8%), and the combination of 14-deoxy-andrographolide (**2**) and 14-deoxy-11,12-didehydroandrographolide (**3**) (17.8 mg, 98.5%, the molar ratio of **2**:**3**, 1:2) were obtained from the 234 mg of injection sample. The yields of andrographolides (2.6% yield of discolored ethanol extract from whole aerial part of Andrographis, and about 40 mg/g of andrographolide in the obtained extracts) are closely consistent with their low contents in the extracts which have been analyzed in previous studies [38,39]. Therefore, the amount of the andrographolide obtained by our method is satisfactory and the yield ratio of the method is relative high, which resulted from the use of support-free counter-current chromatographic purification. One way to obtain larger amount of andrographolides in each CCC run is to use the commerical extracts containing a higher content of andrographolides [24].

4.7. The structural identification

The structures of the products purified by CCC were unambiguously identified as andrographolide (**1**) corresponding to peak (**1**), and a combination of 14-deoxy-andrographolide (**2**), and 14-deoxy-11,12-didehydroandrographolide (**3**) with a molar ratio of 1:2 corresponding to peak (**2**,**3**) by comparison of the spectral data including ESI-MS and 1D and 2D NMR. Although there are some overlapped signals in its 1D ^{1}H and ^{13}C NMR spectra, the 2D ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC spectra clearly indicate that the peak (**2**,**3**) is a two-component combination, from which complete assignments of ^{1}H and ^{13}C NMR of each obtained component were obtained. Meanwhile, ^{1}H NMR spectrum showed distinctly that the molar ratio of component **2** and **3** is 1:2, corroborated by different area ratios of **2** and **3** in HPLC analysis (Fig. 3). In addition, ^{1}H and ^{13}C NMR spectra strongly supported the HPLC analyses that the purities of the obtained andrographolides were good. The detailed NMR data are summarized in Table 4. The positive ESI-MS spectra of all products showed prominent ions of $[M+Na]^+$, and high-resolution ESI-TOF-MS spectra also showed similar fragmented ions and ions from the individual components of the mixture. As showed in Table 5, all MS spectra are in close agreement with their molecular structures. Together with previously reported data [33,40,41], the structures of the products have been established unambiguously as illustrated in Fig. 1.

5. Conclusions

In this work, the theory and application of an overlapping elution–extrusion CCC method has been established for the first

time. As summarized in Fig. 6, a general protocol for the overlapping elution–extrusion CCC preparation of natural product targets often involves several steps, such as (1) extraction of natural products, (2) determination of partition coefficients and selection of a solvent system, (3) pre-determination of retention volumes of stationary phase and mobile phase, and pre-estimation of retention of the target, (4) determination the appropriate switch volume V_{CM} and the next injection time V_j, and (5) performing an elution–extrusion CCC separation. In general, the retention volume of target compounds in the CCC separation can be pre-determined by calculations, and thus the CCC process becomes predictable. The present CCC process provides a new way to prepare repeatedly the cytotoxic natural diterpenoid combination of 14-deoxy-andrographolide and 14-deoxy-11,12-didehydroandrographolide with a molar ratio of 1:2 as well as pure andrographolide for the first time from *A. paniculata*. This overlapping elution–extrusion CCC process with the use of an accurate on-demand liquid phase preparation [32] has been shown to save time and solvent consumption. In practice, the extrusion does not have to be performed at the same flow rate and can be conducted at a much faster rate, saving further time in the separation cycle. Thus, the separation time could be further reduced by selectively increasing the flow rate. The overlapping elution–extrusion CCC is a valuable strategy for facing the current energy and environmental challenges in the global lab.

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