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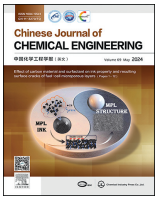

Full Length Article

Simultaneous purification of minor components in natural products using twin-column recycling chromatography with a step solvent gradient

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ABSTRACT

The isolation of minor components from complex natural product matrices presents a significant challenge in the field of purification science due to their low concentrations and the presence of structurally similar compounds. This study introduces an optimized twin-column recycling chromatography method for the efficient and simultaneous purification of these elusive constituents. By introducing water at a small flowing rate between the twin columns, a step solvent gradient is created, by which the leading edge of concentration band would migrate at a slower rate than the trailing edge as it flowing from the upstream to downstream column. Hence, the band broadening is counterbalanced, resulting in an enrichment effect for those minor components in separation process. Herein, two target substances, which showed similar peak position in high performance liquid chromatography (HPLC) and did not exceed 1.8% in crude paclitaxel were selected as target compounds for separation. By using the twin-column recycling chromatography with a step solvent gradient, a successful purification was achieved in getting the two with the purity almost 100%. We suggest this method is suitable for the separation of most components in natural produces, which shows higher precision and recovery rate compared with the common lab-operated separation ways for natural products (thin-layer chromatography and prep-HPLC).

1. Introduction

Natural products refer to the metabolites with special activity that are isolated and extracted from animals, plants and microorganisms in nature [1]. Since the active pharmaceutical ingredients in natural products have been selected and optimized in long evolutionary process, they can be used for the treatment of diseases with good biological activity and targeting ability [2]. At this opinion, the basic research of natural products separation is of great significance for the discovery of leading compounds and development of new drugs [3,4].

Thin layer chromatography (TLC) is the most commonly used method for extracting substances from natural products, which has the advantage of being simple and fast [5]. However, its separation efficiency can be easily influenced by environmental conditions, leading to poor precision and reproducibility [6–8]. In terms of preparative high performance liquid chromatography (HPLC), it is unable to meet the demands for the preparation of substances in research quantities [9–11]. Therefore, there is currently a lack of a universal and efficient method for isolating the desired components from complex natural products, which requires a substantial amount of material for study.

Recycling chromatography is a highly effective purification technique, particularly in the field of separating complex mixtures [12–15]. By repeatedly cycling the sample between separation columns, this method offers improved resolution and sensitivity compared to those single-pass chromatographic methods. For example, a steady-state recycling liquid chromatography system was reported for the recovery of monosaccharides from lignocellulosic hydrolysates, of which separation efficiency was enhanced by 13%–20% [16]. But when it comes to separating minor components from complex natural products, traditional recycling chromatography still has its limitations.

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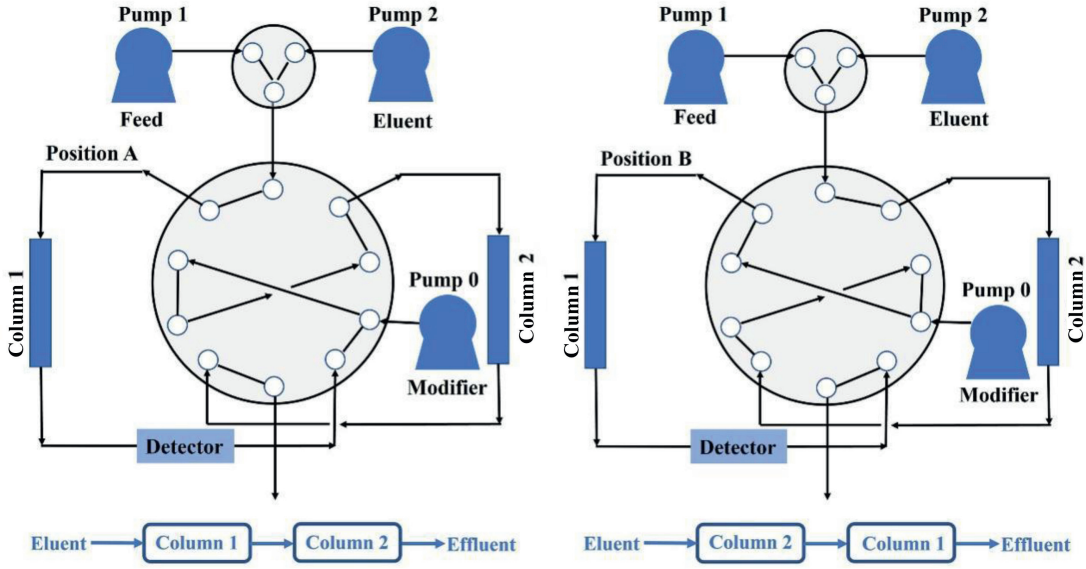

Fig. 1. The schematic diagram of twin-column recycling chromatography with solvent gradient.

The main difficult is the dilution of these minor components due to the band broadening in the process of cycling separation [17,18]. Since the diffusion of molecules from high concentration region to low concentration region is spontaneous, the minor components would be thinning in recovery sample after several cycles and hard to be extracted [19–21]. Therefore, a band compression effect is required to facilitate an enrichment process for the separation of these minor components.

In previous studies, we found the shrinkage of concentration band can be attributed to the reduction of the migration rate along the flowing direction at each concentration point [22,23]. Based on the idea, a twin-column recycling chromatography with a step solvent gradient was proposed to verify its separation effect towards the separation of minor components in natural produces. Two substances with minor contents c1 and c2 in crude paclitaxel were selected as target components. The experiments were divided into two steps. In the first step, methanol/water was used as eluent to separate the crude c1 from other compounds and getting product c2 with high purity. In the second step, the crude c1 was further purified using acetonitrile/water as eluent.

The substance c1 and c2 with a purity of more than 95% were obtained. It should be mentioned that the purification of substance c1 and c2 would not be succeed without the injection of a small amount of water between the two columns, which could effectively offset band spreading, causing the trace target compounds to be concentrated and isolated simultaneously [18]. This work provided a lab-operable method for the preparation of components with close characteristics from natural products in a quick way.

2. The Operating Principle of Twin-Column Recycling Chromatography With a Step Solvent Gradient

The schematic diagram of twin-column recycling chromatography system with a solvent gradient is described in Fig. 1. In position A, the mobile phase is flowing from column 1 to 2, and vice versa in position B. The state of position is switched through the valve. Hence, with the switch of valve, the sample to be separated could be kept flowing in the two columns. After several switches, a satisfactory separation between the target compounds and other substances might be reached. The qualified fractions of the target component are recovered by peak-shaving.

In a typical chromatographic separation, the band broadening is unavoidable, which would dilute concentration of the band (Fig. 2(A)). In order to prevent the attenuation of minor component band in recycling, the modifier pump would work continuously in the whole separation process. As described in Fig. 2(B), with the introduction of water, a solvent gradient between the two columns was created, of which the mobile phase in downstream column is more polar. Thus, the leading edge would migrate slower than the trailing edge when the band is flowing from upstream to downstream column, resulting in the squeeze and contract of the band.

3. Materials and Methods

3.1. Materials

The crude paclitaxel was provided from Zhejiang Dade Pharmaceutical Group Co. Ltd. (China). Methanol (analytical grade, purity $\geq$ 99.5%) was purchased from China Sinopharm Chemical Reagent Group Co. Ltd. (China). Acetonitrile (analytical grade, purity $\geq$ 99.5%) was bought from McLean Reagent Co. Ltd. (China). Pure water was supplied by Wahaha Group Co. Ltd. (China). Before the separation experiments, an amount of 1.25 g crude paclitaxel

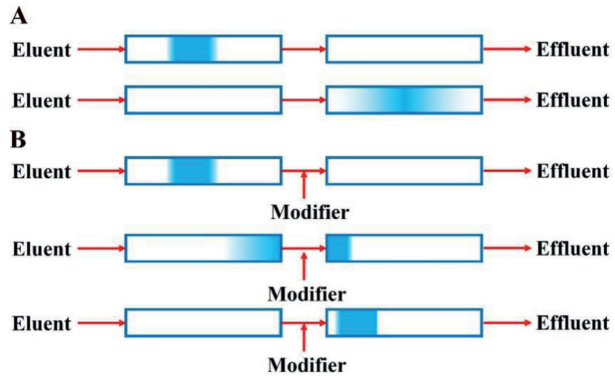

Fig. 2. (A) The band broadening effect in chromatographic separation. (B) The band compression effect caused by the step solvent gradient.

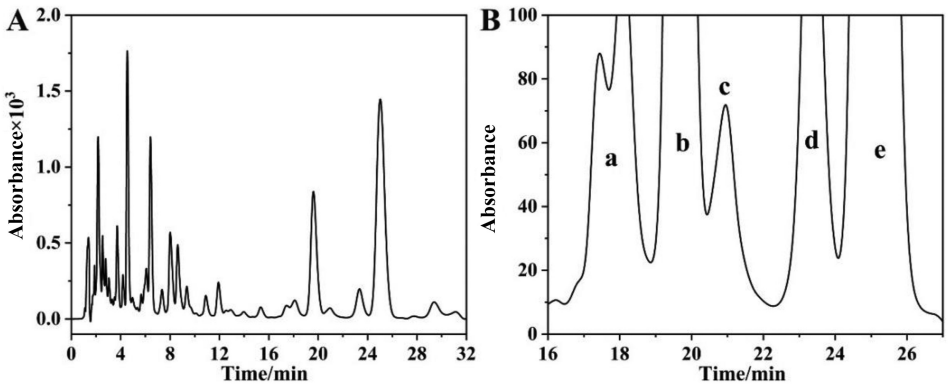

Fig. 3. The HPLC chromatogram of paclitaxel crude (A), and its signal amplification (B). The temperature was set at 40 °C.

was dissolved in 100 ml methanol/water (70/30, volume fraction) solution as the feeding.

3.2. Instruments

The twin-column recycling chromatography system with a solvent gradient consisted of one two-position 10-way valve (Vicivalco, Switzerland), two EPA-C18 silica gel columns (15 μm, 10 nm, 10 mm × 150 mm) and three high-pressure constant-flow pumps (YM1205PHB1, Beijing Qinfang Technology Co., ltd, China) for the delivery of eluent, feed and modifier, respectively. An ultraviolet detector (LC-3068, Beijing Qinfang Technology Co., ltd.) was connected in the outlet of column 1 to follow the real-time separation process.

3.3. Analysis

The qualitative and quantify analyses were performed by using iChrom 5100 high performance liquid chromatography system (HPLC, Dalian Elite Analytical Instruments Co. Ltd., China) with a Phenomenon Kinetex(R) XB-C18 column (5 μm, 4.6 mm × 150 mm). A mixture of acetonitrile/water (40/60, volume fraction) was used as mobile phase at the flow rate of 1.0 ml·min^{-1}. The detection wavelength was 227 nm. The HPLC chromatogram of crude paclitaxel is shown in Fig. 3(A). The target isolate was the substance c peaked at 20.9 min (Fig. 3(B)). Based on the analysis of HPLC, the total content of substance c was about 1.8% in the drug.

3.4. Designing the separation process

As shown in Fig. S1 (in Supplementary Material), the signal curves of the feeding solution flowing through a single column were recorded in using different mobile phases, each with a feeding volume of 5 ml for retention volume (V_R) determination. On the basis of fraction composition analysis after effluent cutting, the elution curve of each substance can be mapped, and the retention volume, which is defined as the volume of eluent that must flow through the column for elution of the target component, is measured. Accordingly, the switching interval (Δt) could be determined on the basis of following inequalities:

$$F_1 \cdot \Delta t \geq V_{R1} \quad (1)$$

$$F_2 \cdot \Delta t \leq V_{R2} \quad (2)$$

where, F_1 and F_2 are the volumetric flow rates of the upstream and downstream liquid, respectively. V_{R1} and V_{R2} are the retention volumes of the target component in the upstream and the downstream columns, which were depended on the liquid compositions. Hence it would be crucial to meet the conditions that both inequalities were established by the adjustment of the modifier composition or flow rate F_M.

In mapping the elution curve of substance c, two peaks with a certain separation degree were existed when using both acetonitrile/water and methanol/water solution as mobile phases, suggesting there should be two compounds peaked at 20.9 min in HPLC chromatogram (Fig. 4). According to the elution order, the two were denoted as substance c1 and c2 respectively. The retention volumes of substance c1 and c2 were measured to be 35 ml and 42 ml when using methanol/water (70/30, volume fraction) as eluent (Fig. 4(A)). When using acetonitrile/water (50/50, volume fraction) as eluent, the retention volume are 40 ml and 47 ml respectively (Fig. 4(B)).

In addition, the retention volumes of the substances that might be retained similar to substance c were also analyzed. As described in Fig. 4(A), substance a and e did not show obvious difference in the retention volume to substance c1 when using methanol/water as eluent, suggesting the difficulties in the separation of substance c1 from these compounds. But when using acetonitrile/water as eluent, a satisfactory gap between peak c1 and other peaks can be observed (Fig. 4(B)). Therefore, a plan with two separation steps was designed for the removal of those non-target compounds and purification of substances c1 and c2 with high purity from crude paclitaxel.

In the first step, methanol/water (70/30, volume fraction) was selected as mobile phase at a flow rate of 4 ml·min^{-1} for initial enrichment of substance c1 and purification of substance c2. The enriched substance c1 was then further purified using acetonitrile/water (50/50, volume fraction) as the mobile phase in the second step. Referring to the column pressure, the flow rate of eluent was set at 3 ml·min^{-1} in this step.

In the whole separation process, a small amount of water was introduced to create a solvent gradient between the two columns for the purpose of better separation. Based on our study, if the solvent gradient was small, the compression effect on the band would not be enough to counter the band broadening, resulting in the leakage of target component from the downstream column outlet during the switch process. But when the modifier flow rate was high, the total bands would shrink excessively, which was not helpful for the separation of target component and impurities, leading to an increasing loss during the cutting [24]. According to the previous experimental experience, we suggested the modifier flow rate of 0.15 and 0.1 ml·min^{-1} in the first and second step were appropriate.

Then, the switching interval can be confirmed. Taking the first step separation as example, the band c would enter into the

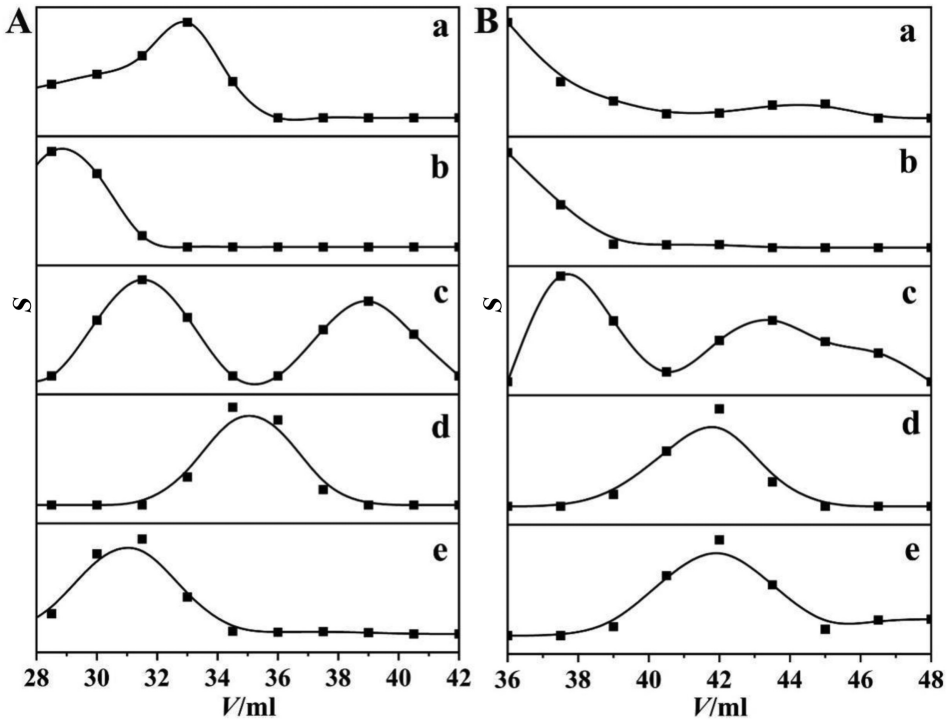

Fig. 4. The peak areas of substance a, b, c, d and e changing with elution volume: (A) mobile phase 70/30 methanol/water, (B) mobile phase 50/50 acetonitrile/water.

downstream column from upstream column completely after 10.5 min based on the inequality 1. However, this period of time is not enough for band c reaching the outlet of downstream column, since the retention volume of substance c increases along with water content in downstream column. Hence the switching interval in the first step was decided to be 10.5 min. The main experiment conditions are listed in Table 1.

In order to verify the effect of separation experiments, the yield (Y) of substance c1 and c2 were calculated with Eq. (3), where S_{Feed} and $S_{Product}$ are the chromatographic peak area of substance c in feed and product solution respectively, and V_{Feed} and $V_{Product}$ are the volume of the feed and the product, respectively. Also, the purity of substance c1 and c2 in the product fraction (Pu) could be determined by normalizing the chromatographic peak area of the product sample.

$$Y = \frac{S_{Product} \times V_{Product}}{S_{Feed} \times V_{Feed}} \quad (3)$$

4. Results and Discussion

4.1. The separation of substance c1 and c2

The signal curve of the separation in the first step is shown in Fig. 5(A), which could be divided into two parts. Part 1 includes switches 1–5 which corresponds to the enrichment of crude c1 and part 2 includes switches 6–9 corresponding to the further purification of substance c2. As described in Fig. S2, the band c was flowing from upstream to downstream column in switch 1 after feeding. In this period, the effluent mainly included substance b and e, which were the pre-impurities. After 10.5 min, the two columns switched positions quickly, at which point the post-impurities, which has a strong adsorption effect on adsorbent in columns, were left in the downstream column and would flow out through effluent (Fig. S3).

After switching for 4 times, the concentration bands of target products c1 and c2 were fully divided. Therefore, the purpose in switch 5 was to collect substance c1 and keep substance c2 in the chromatography system for further purification. In this switch, the effluent was collected by peak shaving at detector outlet once the signal intensity increased to 2800 (Fig. S5). The elution fractions containing substance c1 were denoted as qualified fractions (streamers 2–6) (Fig. 5(B) and Table 2). Referring to the analytical HPLC chromatogram, the content of substance c1 in feeding solution was calculated to be 1.015%.

Based on the HPLC chromatogram, no substance c1 in effluent during switching was detected, suggesting 10.5 min was a fitting switching interval with a solvent gradient between the two columns, which could ensure substance c does not leak from the downstream column. The peak areas of substance c1 at each streamer component are proposed in Fig. 5(C). Those streamers

Table 1
The main experiment conditions in the separations processes

Step	1	2
Purpose	Purification of c2	Purification of c1
Eluent	Methanol/water (70/30, volume fraction)	Acetonitrile/water (50/50, volume fraction)
Eluent flow rate/ml·min^{-1}	4	3
Modifier	Water	Water
Modifier flow rate/ml·min^{-1}	0.1	0.15
Feeding volume/ml	20	16
Switching interval/min	10.5	14
Number of switches	8	8

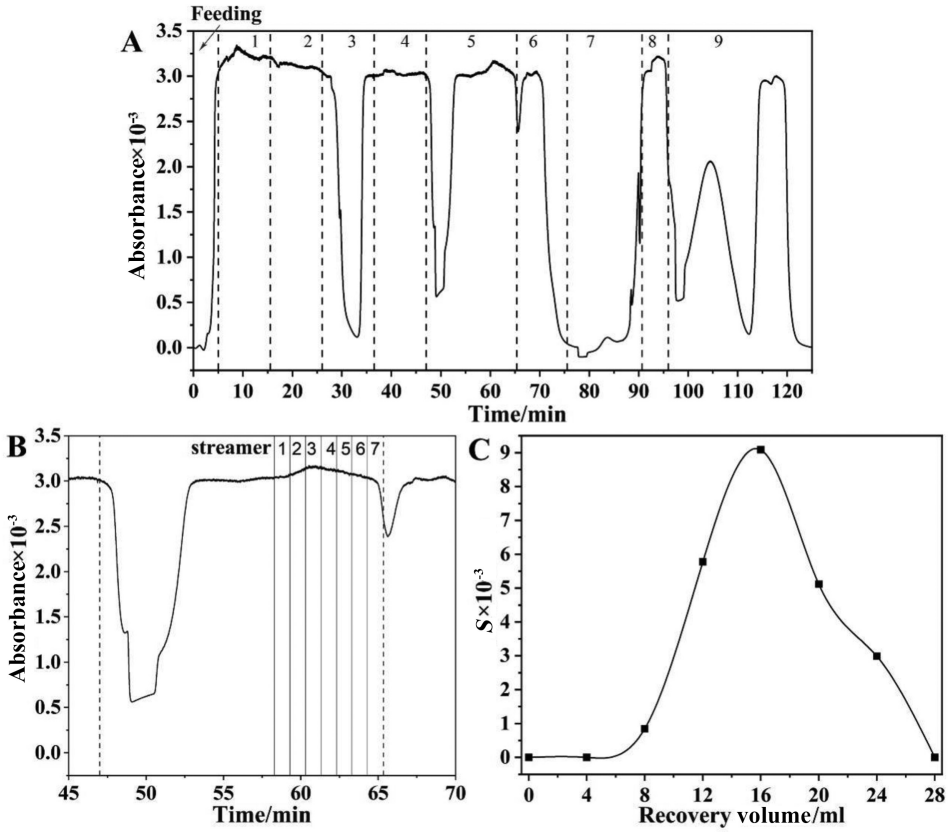

Fig. 5. (A) The elution curve of substance c1 and c2 separation in first step. (B) The time-scale amplification of switch 5. (C) The peak area of substance c1 changing with recovery volume.

Table 2
Volume and substance c1 yield of each streamer in switch 5

Streamer	1	2	3	4	5	6	7
V_{out}/ml	4	4	4	4	4	4	4
Y/%	0	3.5	24.3	38.2	21.7	12.3	0

whose peak area of substance c1 were not less than 1000 were lumped as crude c1 with a volume of 16 ml for further purification process.

4.2. The purification of substance c2

As described in Fig. 5(A), the exist of peak valley between 50 and 60 min should be contributed to the divide of band c1 and c2. Accordingly, the position of the two columns was switched when the signal value decreased to 2800 in switch 5, converting band c2 into the upstream column after the band c1 was flowed out and collected. Since the most of non-target substances had been separated and flowed out in the collection of crude c1, the signal

Table 3
Volume, substance c2 yield and purity of each streamer in recovery

Streamer	1	2	3	4	5
V_{out}/ml	2	2	2	2	2
S	4662	7650	8384	5536	2086
Y/%	16.6	27.1	29.7	19.6	7.0
Pu/%	100	95.0	97.9	79.3	22.9

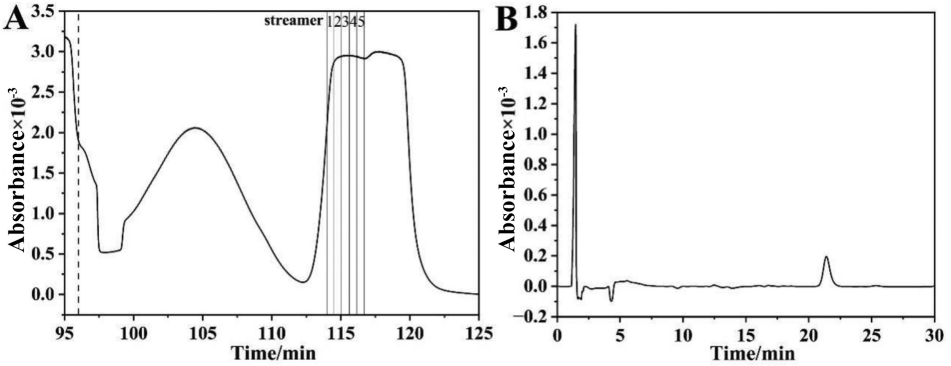

Fig. 6. (A) The time-scale amplification of switch 9. (B) HPLC diagram of product c2.

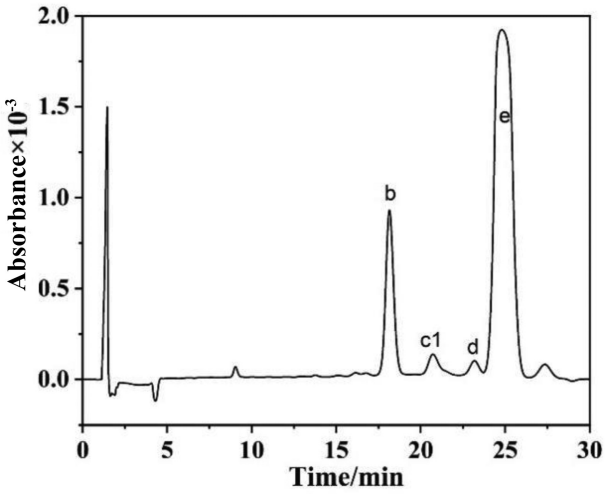

Fig. 7. HPLC chromatogram of crude product c1.

intensity could be the hint showing the movement of target band c2 in part 2. Therefore, the switching time in this part was more flexible, which could be adjusted dynamically according to the signal value of UV detector.

As described in Fig. S5, in switch 6, the band c2 migrated from column 1 to column 2. In this switch, when substance c2 had totally accessed to column 2, the signal value would decrease to zero. Then the columns switched immediately, keeping band c2 in the up-stream column for separation. In switch 7, the band c2 migrated from column 2 to column 1. The low signal value suggested the effluent did not contain our target compound. When the value increased to 2800, the system would switch again, preventing substance c2 flowing out of the chromatograph. After 4 switches in

Table 4
Volume and substance c1 purity of streamer in fraction 2

Streamer	9	10	11	12	13	14	15
V_{out}/ml	1.5	1.5	1.5	1.5	1.5	1.5	1.5
S	3608	6265	8023	10463	9861	8332	3850
Pu/%	52	95	75	63	67	60	42

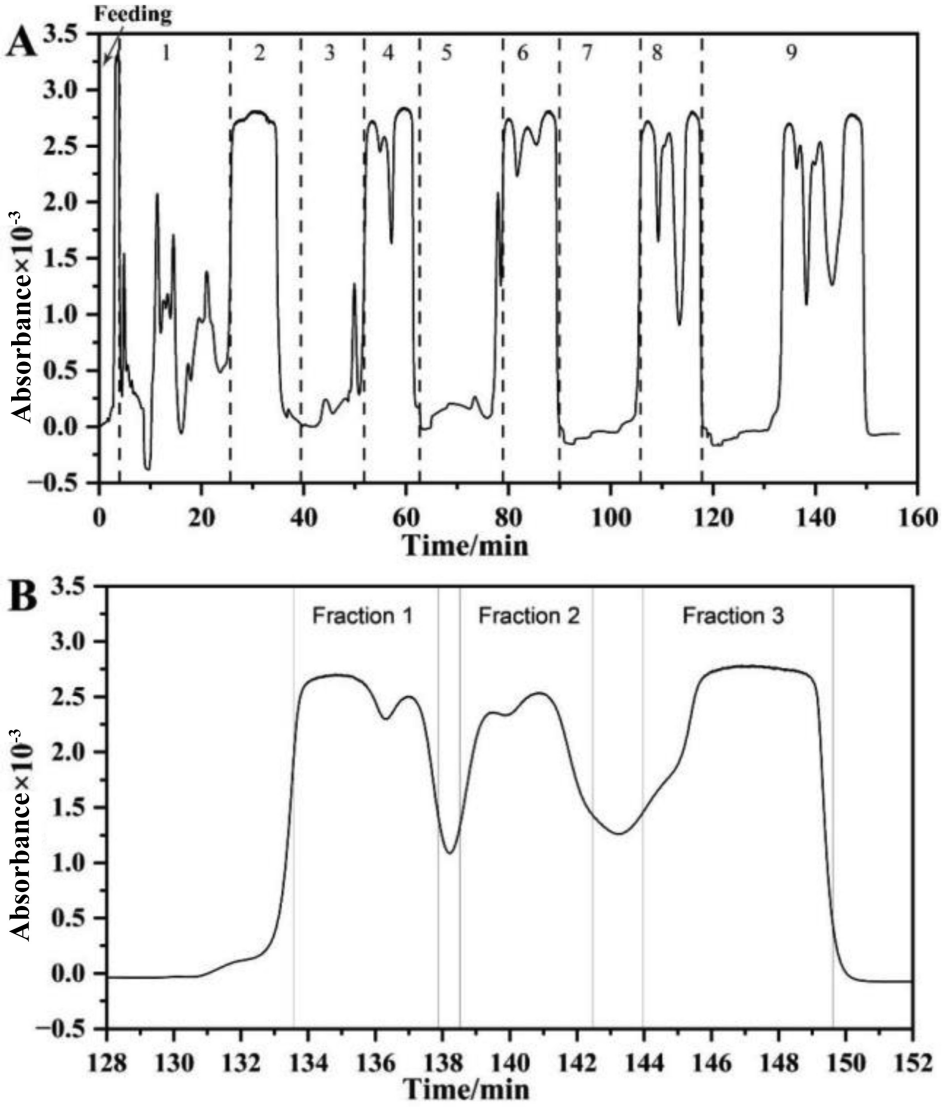

Fig. 8. (A) The signal curve of substance c1 separation in second step. (B) The time-scale amplification of process 9. In this process, 23 pieces of 1.5 mL streamers were extracted and recovered in sections. The recovery effluent can be divided into 3 fractions. Fraction 1 included streamer 1–8, fraction 2 included streamer 9–15, fraction 3 included streamer 16–23. Based on the HPLC of each streamer, substance c1 was in the fraction 2.

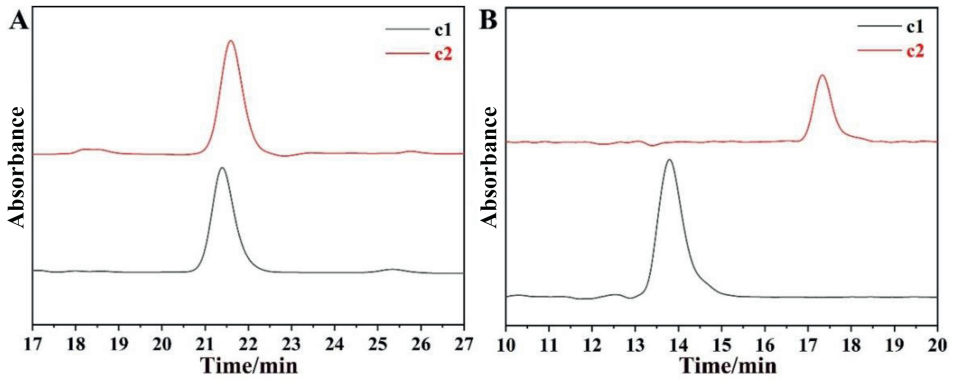

Fig. 9. Chromatogram of quality products c1 and c2 (A) mobile phase: 40/60 acetonitrile/water, (B) mobile phase: 60/40 methanol/water).

part 2, the streamers were extracted and recovered in sections (Fig. 6(A)).

Experimental results in the purification of substance c2 are listed in Table 3. With the increase of recovery volume, the content of substance c2 in streamers first risen and then fallen. The streamers with purity more than 95% were classified as qualified product (Fig. 6(B)). The yield of substance c2 in qualified product was calculated to be 73.4%. As for the effluent loaded in the position of 105 min in Fig. 6(A), the main component was substance e (Fig. S6). And the main component in the effluent after 117 min was also detected to be substance d (Fig. S7). Therefore, in this step, substance c1 and c2 were divided and collected in just one separation process, and a further purification process was required for substance c1.

4.3. The purification of substance c1

The aim in second step was the purification of crude c1 collected in the first step. As shown in Fig. 7, the content of substance c1 in crude product was about 1.9%. In this purification, acetonitrile/water (50/50, volume fraction) was performed as eluent. The valve controlling the state of system was switched according to the signal value to ensure that the substance c1 do not leak out of the downstream column due to excessive elution.

The complete separation process can be described from the signal value curve. As shown in Fig. 8(A), after 8 times of switching, the primary band was divided, the concentration bands of substance c1, b and e were almost separated. Switch 9 was the recovery period, and the detector was connected with the outlet of chromatography system in this switch. Based on the change of signal value, three fractions containing 23 pieces of 1.5 ml streamers were extracted and recovered in sections (Fig. 8(B)). The effluent with low signal intensity (less than 2000) were recorded as waste, in which was not detected substance c1. According to the HPLC analysis, the streamers with purity more than 95% were combined as product (Table 4). The yield of substance c1 was calculated to be 12%.

The purities of the products c1 and c2 were verified based on the HPLC chromatogram after separation. As described in Fig. 9(A) and (B), the purities of quality product c1 and c2 were calculated to be 95.3% and 99.1% respectively when using acetonitrile/water solution as mobile phase. In addition, when using menthol/water as mobile phase, they were 98.1% and 99.5% respectively. The different retention times of substances c1 and c2 in the methanol/water condition could also be the evidence that there are two different substances in peak c. In brief, the products with purity more than 95% were gotten by using twin-column recycling chromatography system with a step solvent gradient in this work.

5. Conclusions

In order to investigate the isolation performance of minor components from complex natural products using twin-column recycling chromatography system with a solvent gradient, two substances, c1 and c2, whose total contents in paclitaxel were less than 1.8%, were selected as the target components for purification. In the conditions of methanol/water and acetonitrile/water as mobile phases, both substances were successively separated, with purity over 95%. This work testified the effectiveness of band compression mechanism triggered by solvent gradient, overcoming the problems of low separation efficiency in the purification of active pharmaceutical ingredients in natural products. After removing water and organic solvent, the target components can be used for quantitative and qualitative analysis directly. This separation system is beneficial to improve the level of natural product research and contribute to the quality and safety control of drugs.

CRediT Authorship Contribution Statement

The first author Dr. Guangxia Jin carried out the experiments and wrote the first draft of the manuscript. Dr. Yuxue Wu were involved in planning the work and performed the data analysis. The corresponding author Prof. Feng Wei was in charge of the overall direction and planning. He was also involved in the revision of the manuscript.

Declaration of Interest

The authors declare no conflict of interest.

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Supplementary Material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cjche.2024.02.004.

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