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Separation of honokiol and magnolol by intermittent counter-current extraction

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ABSTRACT

Recently, intermittent counter-current extraction (ICcE) has been developed and shown its advantage in improving resolution between targeted compounds. However, how to choose suitable parameters to increase the throughput has not been systematically studied yet. In present work, we first calculated theoretically the conditions to carry out ICcE elution mode. Then, honokiol and magnolol were separated as model compounds using ICcE elution mode to confirm our conclusion. After parameters like sample concentration and sample feed were optimized in analytical high-performance counter-current chromatography (HPCCC), the separation process was scaled up to preparative HPCCC successfully. 12.8 g honokiol and 16.1 g magnolol were separated from 30 g mixture with purities of 98.6% and 93.7%. And the throughput of target isolation of ICcE elution mode was at least 3.75× higher than isocratic elution mode with the same HPCCC instruments. Our results confirmed our theory calculation and demonstrated the enormous potential of ICcE on preparative separation of binary mixture.

1. Introduction

High-speed counter-current chromatography (HPCCC) is a steady and repeatable preparative liquid–liquid partition separation method [1–3]. The character of liquid stationary and mobile phase makes it easy to pump one phase in the opposite direction to the other at the same time and to supply sample continuously to the center of coil, which is called continuous counter-current extraction (CCcE) [4,5]. CCcE can improve throughput, but it requires a special coil. Recently, Hewitson et al. reported a new operational scenario for conventional two-bobbin CCC centrifuges, called intermittent counter-current extraction (ICcE). In ICcE, upper phase and lower phase are pumped into the coils as a mobile phase alternately, and sample is injected between two coils [6,7].

However, up to now, there is no report on how to choose suitable separation conditions such as partition coefficient (K_D values) of compounds and retention of stationary phase (S_f) in ICcE. In the present work, we reported a simple strategy to set S_f and to choose suitable K_D values through theory calculation. Then, honokiol and magnolol were separated as model compounds using ICcE elution mode on analytical HPCCC instrument. After parameters like sample concentration and injection speed were optimized on analytical column, the separation process was successfully scaled up to preparative HPCCC. 12.8 g honokiol and 16.1 g magnolol were separated from 30 g mixture with purities of 98.6% and 93.7%. Our results confirmed our theory calculation and indicated that the throughput of ICcE was at least 3.75× higher than that of isocratic elution mode with the same HPCCC instruments.

2. Experimental

2.1. Apparatus

All separations were performed on a Midi-DE HPCCC system (Dynamic Extraction Ltd., Slough, UK). The apparatus was equipped with four coils on two bobbins—an analytical and preparative coil on each bobbin. The analytical coils are stainless steel tubing of 0.8 mm diameter with column volumes for coils 1 and 2 being 17.0 and 16.0 ml respectively. The preparative coils are 4 mm Polyfluoroalkoxy (PFA) tubing. The volumes of coil 3 and coil 4 are 451.0 and 458.0 ml respectively. The revolution radius for all these coils is 11 cm with a β value varying from 0.52 at the internal terminal to 0.86 at the external terminal. In this study, the rotation speed was kept at 1250 rpm, and the "*g*" value was 215 g. This apparatus was coupled with 3 ÄKTA Plus pumps (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden) which were used to pump two-phase solvent system and sample solution as shown in Fig. 1.

The analytical high-performance liquid chromatography (HPLC) system used throughout this study consisted of Waters 2695XE

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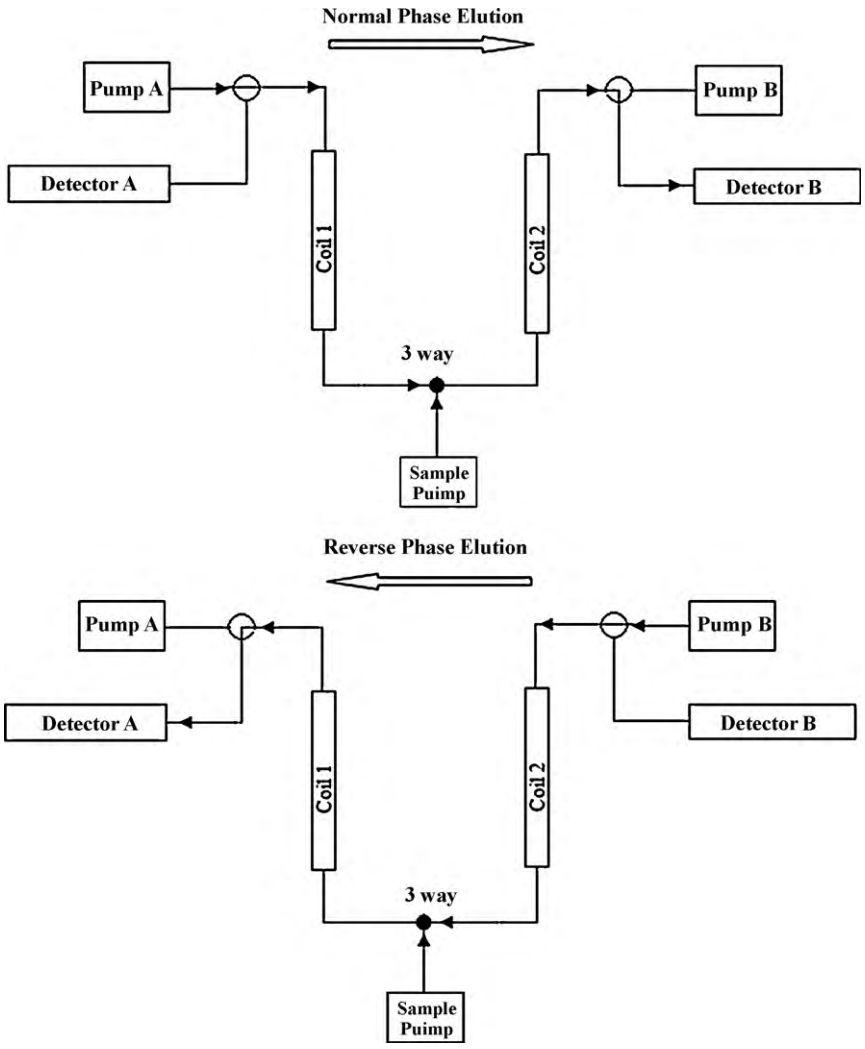

Fig. 1. Intermittent counter-current extraction (ICcE) operation modes.

including a Waters 2487 Dual wavelength Detector (Waters, Milford, MA, USA).

2.2. Reagent

Analytical grade n-hexane, ethyl acetate and methanol for HPCCC separation were purchased from Changzheng Chemical Factory, Chengdu, China. Acetonitrile used for HPLC was chromatographic grade and purchased from Fisher Chemical (Loughborough, UK). Water was produced by Milli-Q system (18 MΩ) (Millipore, Bedford, MA, USA).

The mixture of honokiol (44.5%) and magnolol (55.2%) was obtained from Jiuding Chemical Technical Co. Ltd. (Chengdu, China).

2.3. Preparation of solvent system and sample solution

K_D values of honokiol and magnolol in different solvent system were studied systematically in our previous work [8]. Hexane–ethyl acetate–methanol–water (2:1:2:1) with K_{Dh} of 0.67 and K_{Dm} of 1.31 was chosen as a solvent system. The solvent mixture was equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The mixture of magnolol and honokiol was dissolved in equal volume upper and lower phases.

2.4. Calculation in theory

In recent research [7], Yang et al. constructed a mathematical model of ICcE. In this model, the linear velocity of solute in two elution direction could be calculated with Eqs. (1) and (2):

$$\mu_{X.L} = \frac{(F_L/A_C)(\beta_L + 1)}{1 + K_D\beta_L} \tag{1}$$

$$\mu_{X.U} = \frac{(F_U/A_C)K_D(\beta_U + 1)}{1 + K_D\beta_U} \tag{2}$$

where $\beta_L = A_{U.L}/A_{L.L}$ (volume ratio of two phases when lower phase as mobile phase) and $\beta_U = A_{U.U}/A_{L.U}$ (volume ratio of two phases when upper phase as mobile phase). Then the average linear velocity of the solute peak is given by

$$\begin{aligned}\mu_{X,i} &= \frac{\mu_{X.l}t_{i.L} - \mu_{X.U}t_{i.U}}{t_{i.L} + t_{i.U}} \\ &= \frac{(F_L/A_C)t_{i.L}(\beta_L + 1)/(1 + K_D\beta_L) - (F_U/A_C)t_{i.U}K_D(\beta_U + 1)/(1 + K_D\beta_U)}{t_{i.L} + t_{i.U}}\end{aligned} \tag{3}$$

where $t_{i.L}$ and $t_{i.U}$ indicate the unit programmed time for forward and back ward elution, respectively.

In the present work, for simplifying the equation, we set the retention of stationary phase $S_f = 0.5$ in both elution directions, then $\beta_L = \beta_U = 1$; and $t_{i.L} = t_{i.U}$. Eq. (3) could be expressed as:

$$\mu_{X,i} = \frac{F_L - K_D F_U}{(1 + K)A_C} \tag{4}$$

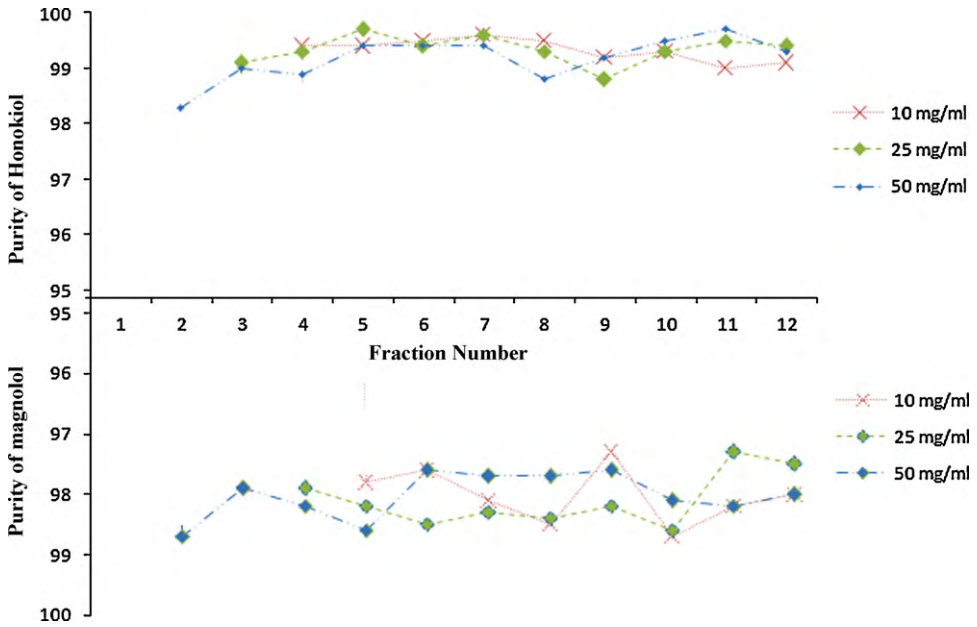

Fig. 2. Results of sample concentration optimization. Solvent system HEMW at (2:1:2:1, v/v); stationary phase: flow rate of lower phase: 1 ml/min; flow rate of upper phase: 1 ml/min; revolution speed: 1250 rpm; separation temperature: 25 °C; Sample concentration 10 mg/ml, 25 mg/ml and 50 mg/ml; sample injection flow rate: 0.1 ml/min; detection wavelength: 280 nm; flow switched every 5 min.

Because we set the $S_f = 0.5$ through the separation process, F_L and F_U were constant and could be obtained from the linear relationship between the square root of flow rate and S_f. If we could choose a suitable solvent system to make the average linear velocity of honokiol $\mu_{(X,i,H)} < 0$, and the average linear velocity of magnolol $\mu_{(X,i,M)} > 0$, honokiol will be eluted by upper phase from the right terminal and magnolol will be eluted by lower phase from the left terminal.

2.5. Measurement of distribution ratio (K_D)

Measurement of K_D values was performed as follows [9]: First, two-phase solvent systems were prepared and equilibrated, and then crude sample (1 mg) was weighed into a 10 ml glass tube and added 1 ml of each phase of a pre-equilibrated two-phase solvent system. After that, the test tube was shaken vigorously until the two phases solvent thoroughly equilibrated. After settling, 100 μl of each phase was transferred to two separate test tubes and condensed under vacuum. The residue was diluted with 1 ml acetonitrile and analyzed by HPLC. The K_D value was expressed as the peak area of target compounds in the upper phase divided by that in the lower phase.

2.6. HPCCC separation

2.6.1. Analytical HPCCC separation procedure

The analytical coils were first filled with the upper phase of solvent system. Then, the apparatus was rotated at 1250 rpm, and at the same time, the lower phase was pumped through the column at a flow-rate of 1.7 ml/min. After hydrodynamic equilibrium was established in the column, the flow-rate of pump A and pump B was set at 1 ml/min, and the cycle of normal phase elution and reversed phase elution (switched every 5 min) started. The temperature was set at 30 °C. The separation process continued 2 h. Fractions eluted from head and tail in each cycle were collected and marked as H1–H12 and T1–T12 respectively. After evaporated under vacuum, residues were dissolved by methanol for HPLC analysis.

2.6.1.1. Sample concentration loading study. 10, 25, 50 and 75 mg/ml of sample concentration were tested. Flow-rate of pump A and pump B was set at 1 ml/min. The injection speed (pump C) of sample solution was kept at 0.1 ml/min.

2.6.1.2. Injection flow rate of sample solution study. According to the results of Section 2.6.1.1, the sample concentration was set at 50 mg/ml. The initial injection speed of sample solution was set at 0.2, then 0.4 and 0.6 ml/min (0.1 ml/min had been tested in Section 2.6.1.1) were tested. Flow-rate of pump A and pump B was set at 1 ml/min.

2.6.2. Preparative HPCCC separation procedure

The preparative separation procedure was similar to Section 2.6.1 except several operation parameters. The flow-rate for equilibrium was 42.5 ml/min for the 50% stationary phase retention. After equilibrium, the flow-rate of pump A and pump B was set at 25 ml/min and the injection speed of sample solution was set at 5 ml/min.

2.7. HPLC analysis of crude extract and HPCCC fractions

The crude extract and fractions separated by HPCCC were analyzed by HPLC coupled with a dual wavelength detector. The HPLC condition was as follows: the column used in present study was a Sunfire C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Waters). The mobile phase composed of acetonitrile–water (60:40, v/v) was eluted at a flow-rate of 1 ml/min. The UV detector was set at 269 nm. The temperature was set at 30 °C.

3. Results and discussion

3.1. Optimization of the flow rate

In Section 2.4, the S_f was set at 50% for simplifying the mathematic model. In this case, the S_f should be kept at 50% through the separation process. The relationship between flow rate and S_f was studied by Du et al. [10], the regression analysis of S_f showed a linear relationship between the square root of the flow-

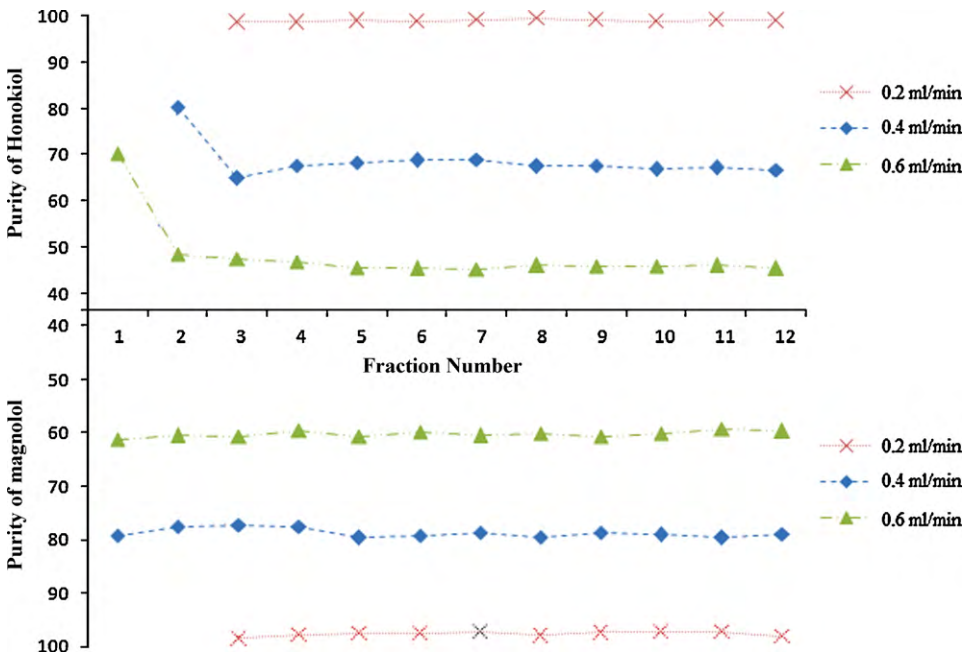

Fig. 3. Results of sample injection flow rate optimization. Solvent system HEMW at (2:1:2:1, v/v); stationary phase: flow rate of lower phase: 1 ml/min; flow rate of upper phase: 1 ml/min; revolution speed: 1250 rpm; separation temperature: 25 °C; sample concentration 50 mg/ml; sample injection flow rate: 0.2 ml/min, 0.4 ml/min and 0.6 ml/min; detection wavelength: 280 nm; flow switched every 5 min.

rate and the retention percentage of the stationary phase. At the beginning of the separation process, upper phase of solvent system was used as stationary phase. Then 0.5, 1, 1.5 and 2 ml/min were selected as flow-rate on analytical coils, and the S_f values were 90%, 72.2%, 57.8% and 45.5%, respectively. The relationship between flow-rate and S_f could be shown as $S_f = -63.09F^{1/2} + 134.8$, $R^2 = 0.999$. Therefore if S_f equals 0.5 then the corresponding flow rate will be 1.7 ml/min.

3.2. Results of parameters optimization on analytical coils

3.2.1. Optimization of sample concentration

The purities of fractions with different sample concentrations are shown in Fig. 2.

When sample concentrations were set at 10, 25 and 50 mg/ml, respectively, honokiol (T1–T12) and magnolol (H1–H12) were separated with high purity. When sample concentration was 75 mg/ml, sample solution could not be dissolved very well. Hence, the optimized sample concentration was 50 mg/ml.

3.2.2. Optimization of injection speed

As shown in Fig. 4, when injection speed of sample was increased to 0.4 ml/min, the purities of honokiol and magnolol decreased sharply. So the optimized injection speed was set at 0.2 ml/min. The purities of fractions with different injection flow rates were shown in Fig. 3

3.3. Scale-up separation

In this study, preparative coils and analytical coils had the same rotation radius and β values, so the parameters optimized on analytical coils could be scaled up to preparative coils linearly [11]. The volume of preparative coils is 25× larger than analytical coils. Hence, the flow-rate of pump A and pump B was set at 25 ml/min, the injection speed of sample solution was set at 5 ml/min. The scale-up process by preparative coil was shown in Fig. 4. After all fractions were collected, the purities of honokiol and magnolol achieved 98.6% and 93.7%, respectively.

3.4. The verification of theory calculation

In intermittent counter-current extraction, Kostanyan et al. described the movement of solute with the following equations [12,13]:

$$Q_L = \frac{1 - C^m}{1 - C^{n+1}} \tag{5}$$

$$Q_U = \frac{C^m - C^{n+1}}{1 - C^{n+1}} \tag{6}$$

where extraction factor $C = F_U K_D / F_L$, n is the total number of equilibrium stages and m is the position of the sample inlet; Q_U is the portion of solute eluted by upper phase, Q_L is the portion of solute eluted by lower phase. When the sample is fed at the middle of the column, $m = (n+1)/2$, and Eqs. (5) and (6) could be written as:

$$Q_L = \frac{1}{1 + C^{(n+1/2)}} \tag{7}$$

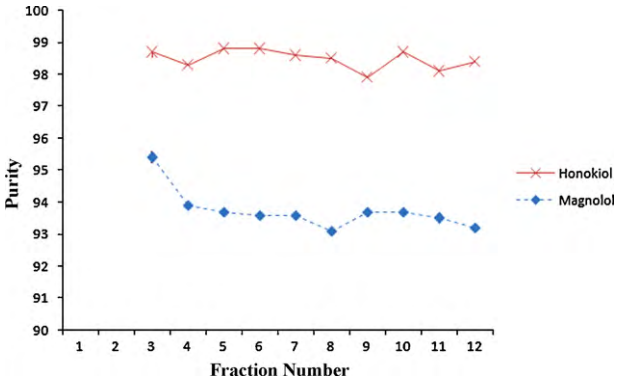

Fig. 4. Results of prep-ICCCE. Results of sample injection speed optimization. Solvent system HEMW at (2:1:2:1, v/v); stationary phase: flow rate of lower phase: 25 ml/min; flow rate of upper phase: 25 ml/min; revolution speed: 1250 rpm; separation temperature: 25 °C; sample concentration 50 mg/ml; sample injection flow rate: 5 ml/min; detection wavelength: 280 nm; flow switched every 5 min.

Table 1
Comparison between isocratic elution mode and intermittent elution on analytical HSCCC.

Elution mode	Sample concentration (mg/ml)	Sample volume (ml/h)	Sample mass (mg)	Purity of honokiol (%)	Purity of magnolol (%)
Isocratic (normal phase)	80	2	160	98.6	99.9
Intermittent	50	12	600	98.6	93.7

$$Q_U = \frac{C^{(n+1/2)}}{1 + C^{(n+1/2)}} \quad (8)$$

The number of equilibrium stages can be calculated using Eqs. (7) and (8) from experimental measurements of Q_U and Q_L. In the present work, $F_U = F_L$, then $C_H = K_{Dh} = 0.67$, $C_M = K_{Dm} = 1.31$. From Fig. 2, we could know that the portion of honokiol eluted by upper phase was 0.995. According to Eq. (7), $n = 25$. Using this value, we get the $Q_{UM} = 0.976$ which is in good agreement with the experimental results of Fig. 2.

3.5. The comparison of throughputs between ICcE and isocratic HPCCC

In our previous work, we have reported the separation of honokiol and magnolol by isocratic HPCCC on analytical HPCCC and preparative HPCCC. In this study, we choose honokiol and magnolol as model compounds to perform the same separation with *ICcE* HPCCC. Compared with our previous results [8], 3.75× throughputs were obtained (Table 1) on analytical HPCCC in the present work. And this result was further confirmed on preparative separation with *ICcE* HPCCC, indicating the great advantages of *ICcE* HPCCC over isocratic HPCCC on preparative separation.

4. Conclusion

In this study, we analyzed the condition of carrying out ICcE in theory and parameters such as K_D value and retention of stationary phase were obtained. For testing our calculation, two model compounds, honokiol and magnolol were separated by ICcE with these parameters successfully. The separation process was scaled up to a preparative ICcE. Compared with our previous research, 3.75× throughput of target isolation was obtained. This is the first time to report that the parameters of ICcE are calculated in theory and the throughputs of ICcE and isocratic HPCCC are compared. Our research showed a great potential of ICcE on preparative separation.

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