A Neglected Issue: Stationary Phase Retention Determination of Classic High-Speed Counter-Current Chromatography Solvent Systems

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Abstract: Obtaining an ideal solvent system for target compounds is still an obstacle to the wide application of high-speed counter-current chromatography (HSCCC). The partition coefficient and retention of the stationary phase are two key parameters for solvent system selection. The retention of the stationary phase of the solvent system is roughly judged by settling time using a test tube, which is subjective and inaccurate. In this study, we demonstrated that high-resolution separation of HSCCC is tightly connected with the retention of the stationary phase. Notably, unlike the in vitro test of settling time, we investigated the retention of the stationary phase of classical biphasic solvent systems by a TBE300C HSCCC apparatus. Our results revealed that settling time is not always inversely proportional to the retention of the stationary phase. The \( n \)-hexane–ethylacetate–methanol–\( n \)-butanol–water solvent systems showed the highest correlation coefficient of settling time and retention of the stationary phase (\( r = -0.91, n = 16 \)). \( N \)-heptane–\( n \)-butanol–acetonitrile–water solvent system showed the lowest correlation coefficient (\( r = -0.26, n = 7 \)). These results may be helpful for HSCCC solvent system selection and accelerate the application of this technique.

Keywords: high-speed counter-current chromatography; retention of the stationary phase; settling time; solvent systems

1. Introduction

HSCCC has become a useful tool in separating natural products [1], proteins [2], steroids [3] and antibiotics [4]. This technology has gained great popularity due to its merits, such as time-saving, larger loading and no sample irreversible adsorption. Once an optimal solvent system is selected, the target compounds can be obtained in several hours. Nevertheless, it is a challenging task to find the best solvent system to use HSCCC. Researchers have to spend about 90% of the entire work in HSCCC to obtain a solvent system with suitable K values (\( 0.5 \leq K \leq 2.0 \)) and high resolution [5]. To make this task easier, several two-phase solvent systems with a broad range of hydrophobicity, including the \( n \)-hexane–ethylacetate–methanol–\( n \)-butanol–water solvent systems [6], chloroform–methanol–water systems [7] and \( n \)-heptane–\( n \)-butanol–acetonitrile–water solvent systems [8], have been explored and widely used.

By repetitive partition of the solutes in the two-phase solvent system at a high rate, HSCCC offers good separation efficiency. The volume of retained stationary phase in the coiled column can be considered as a “liquid column”, and the separation efficiency of HSCCC is tightly connected with this parameter. According to Ito, the retention of the stationary phase can be roughly evaluated by measuring the settling time of solvent systems. Usually, if the settling time of one solvent system is less than 20 s, this solvent
system would give ideal retention of the stationary phase, and finally good separation results [6]. Nevertheless, the settling time is somewhat subjective and varies due to the difference of invert time and force, or the volume of tested containers. Therefore, detecting the retention of the stationary phase using the HSCCC apparatus is more reliable than using the settling time, and it is of great importance to know the retention of the stationary phase of different solvent systems (usually no less than 30%). To date, the settling time of various solvent systems have been reported in studies [9], but the values of the stationary phase of different solvent systems are still lacking. Researchers have built a mathematical model to predict the retention of the stationary phase in different operation conditions to promote the application of HSCCC [10]. Additionally, analytical HSCCC is mainly used to choose the best solvent systems with a higher separation efficiency and retention of the stationary phase. Although the results obtained by analytical HSCCC are easy and efficient to scale up on preparative or semi-preparative apparatuses, its cost should be taken into consideration. In this study, we give evidence that the peak resolution of HSCCC is tightly connected with the retention of the stationary phase. Additionally, to give guidance for solvent system selection, the retention of the stationary phase of common classic solvent systems was determined using a TBE-300C apparatus. The results of this study will be helpful in the application of HSCCC.

2. Materials and Methods

2.1. Reagents

All solvents used for the preparation of enriched extract and HSCCC separation were of analytical grade (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China). Methanol used for HPLC was of chromatographic grade (Honeywell Burdick & Jackson, Muskegon, MI, USA), and the water used was distilled water.

2.2. Apparatus

The HSCCC instrument employed in the present study was a TBE-300C high-speed counter-current chromatography instrument (Tauto Biotech, Shanghai, China) with three multilayer coil separation columns (total volume = 300 mL) and a 20 mL sample loop. The revolution speed of the apparatus can be regulated with a speed controller in the range from 0 to 1000 rpm. An HX-105 constant-temperature circulator (Beijing Changliu Instrument Company, Beijing, China) was used to control the separation temperature using water as the circulating media. Continuous monitoring of the effluent was achieved with a model TBD 2000 UV detector. HPLC analysis was performed using high-performance liquid chromatography (Agilent 1220 Infinity II, Agilent Technologies Ltd., Santa Clara, CA, USA), and a ZORBAX SB-C18 (4.6 mm × 250 mm, 5 µm) column at 45 °C. The wavelength for detection was 254 nm.

2.3. Settling Time Evaluation

The settling time of solvent systems was determined according to a previous study [6]. Briefly, 2 mL of equilibrated lower and upper phase, respectively, are added to a test tube (5 mL capacity). After inverting the container 5 times and immediately setting it in an upright position, the time needed to form clear layers between the two phases, called settling time, and is recorded. The experiment was repeated 3 times to obtain the mean value.

2.4. Enrichment of Myricanol from the Bark of Myrica rubra (Lour.) Siebold & Zucc

The bark of M. rubra was air-dried and ground to pass through a 20−mesh screen. Fifteen kilograms of the powder was extracted with 90% ethanol (1:3, v/v) at 35 °C for 12 h, then filtered. Filtrates were evaporated under reduced pressure at 50 °C to afford a brown residue. The ethanol residue was suspended in distilled water and successively partitioned with petroleum ether, ethylacetate, n-butanol and distilled water. After evaporating, each residue was stored at −4 °C until use. The ethylacetate extract (80 g) was first applied to a
D–101 macroporous resin column (2500 g) and eluted with 5 column volumes of 30%, 50%, 70%, 90% and 100% ethanol solution. The enriched myricanol was found in 90% eluent, after evaporation, and this fraction was used for HSCCC separation.

2.5. The Relationship between Resolution and the Retention of the Stationary Phase

To explore the relationship between resolution and the retention of the stationary phase, HSCCC was applied for the separation of myricanol from the ethylacetate extract of the bark of *M. rubra*. The partition coefficient of myricanol in *n*-hexane–ethylacetate–methanol–water (1:1:1:1, v/v/v/v) was 1.40, so this solvent system was selected for further investigation. The rotation speed was set at 850, 400 and 180 rpm, respectively, to obtain different retentions of the stationary phase. Then, the resolution of the separation results was calculated using the following equation:

\[
Rs = \frac{2(T_1 - T_2)}{(W_1 + W_2)},
\]

where \(T_1\) and \(T_2\) are the retention time of the first and second eluting compound, and \(W_1\) and \(W_2\) are the corresponding peak widths at baseline.

2.6. Measurement of the Retention of the Stationary Phase

A set of two-phase solvent systems with a broad range of hydrophobicity were prepared by adding corresponding solvents to a funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. The upper phase and the lower phase were separated before use. The retention of the stationary phase (RSP) was determined by the following procedure: a coiled column was first filled with the stationary phase (upper phase), and then the apparatus was rotated at 850 rpm in head–to–tail mode, while the mobile phase was pumped into the column at 3 mL min\(^{-1}\). The separation temperature was set at 30 °C using a constant-temperature circulator. After the mobile phase flowed out from the column and hydrodynamic equilibrium was established in the column, the eluted volume of the stationary phase was measured, and the volume of remaining stationary phase in the coiled column could be calculated by subtracting the eluted volume of the stationary phase from the total volume of the apparatus. The retention of the stationary phase of each two-phase solvent system was repeated three times.

3. Results

3.1. Separation of Myricanol from the Ethylacetate Extract of the Bark of *M. rubra* and the Relationship between Resolution and the Retention of the Stationary Phase

According to our preliminary study, the *n*-hexane–ethylacetate–methanol–water solvent systems were chosen for the separation of myricanol from ethylacetate extract of the bark of *M. rubra*. The partition coefficient of myricanol in *n*-hexane–ethylacetate–methanol–water (1:1:1:1, v/v/v/v) was 1.40, and this value of another unknown compound is 0.53. So, this solvent system was used for HSCCC separation and the HSCCC chromatogram is shown in Figure 1A. Two hundred and eighty milligrams of 90% eluent of ethylacetate extract of the bark of *M. rubra* was dissolved in 4 mL upper and 4 mL lower phase and yielded 56.30 mg myricanol (eluted out between 98 and 118 min) upon recrystallization from ethylacetate. The retention of the stationary phase was 71.48% at rotation speed 850 rpm. The HPLC analyses of the crude extract from the bark of *M. rubra* and purified myricanol are shown in Figure 1D. The \(^1\)H and \(^13\)C NMR spectroscopic data can be found in Supplemental Figure S1. To explore the relationship between resolution and the retention of the stationary phase, the other operating conditions such as separation temperature, flow rate and loading sample amount were fixed, and the rotation speed was decreased from 850 to 400 and 180 rpm, respectively. As shown in Figure 1B,C, at 400 rpm, the retention of the stationary phase was decreased to 55.7% and the resolution was decreased from 4.6 to 2.9. At 180 rpm, the retention of the stationary phase was further decreased to 36.4% and the resolution was decreased to 1.5. These data indicated that the retention of the stationary phase plays a crucial role in the separation efficiency of HSCCC.
The solvent system for HSCCC is n-hexane–ethylacetate–methanol–water (1:1:1:1, v/v/v/v); flow rate: 3.0 mL min\(^{-1}\); detection wavelength: 254 nm; separation temperature: 30 °C; sample size: 280 mg dissolved in 4 mL upper and 4 mL lower phase. Three rotation speeds (S), 850 rpm (A), 400 rpm (B) and 180 rpm (C), were selected to demonstrate that the resolution (Rs) is tightly correlated with the retention of the stationary phase (RSP). (D) HPLC chromatogram of the pre-purified ethylacetate extract of the bark of M. rubra (upper) and purified myricanol (lower).

3.2. The Retention of the Stationary Phase of a Set of Classical Two-Phase Solvent Systems

It is known that retention of the stationary phase can be affected by many separation conditions, such as temperature, rotation speed, flow rate of mobile phase, as well as viscosity and density difference of the solvent system, etc. [11]. Under most circumstances, the proper range of flow rates of the mobile phase is 1 to 5 mL min\(^{-1}\), and the most widely used rotation speed for HSCCC is 800 or 850 rpm [12]. Therefore, in this study, the rotation speed and flow rates of the mobile phase were set at 850 rpm and 3 mL min\(^{-1}\) to study the retention of the stationary phase of a series of solvent systems.

The n-hexane–ethylacetate–methanol–water systems are capable of separating both hydrophobic and hydrophilic compounds. Chloroform–methanol–water systems are mainly used for the separations of natural products with moderate hydrophobicity. As shown in Tables 1 and 2, the retention of the stationary phase of the n-hexane–ethylacetate–methanol–water system ranges from 60.66% to 74.75%. Normally, a shorter settling time will result in higher stationary phase retention in the column. This study indicated that the value of retention of the stationary phase is not always inversely proportional to the value of settling time. This may be because during the hydrodynamic equilibrium process of HSCCC, repetitive partitioning was happening in the two-phase solvent system at a high rate, which was different from the situation in the test tube. Moreover, variance in operation temperature and force may also affect the results.
Table 1. The retention rate of classic n-hexane–ethylacetate–methanol–water solvent system.

| No | nC₆H₁₄ | EtOAc | nBuOH | MeOH | H₂O | VR (U/L) | ST (s) | MP      | RR (%)  |
|----|---------|-------|-------|------|-----|----------|--------|---------|---------|
| 1  | 10      | 0     | 0     | 5    | 5   | 1.06     | 5 ± 2  | Lower Phase | 73.44 ± 2.15 |
| 2  | 9       | 1     | 0     | 5    | 5   | 0.96     | 9 ± 3  | Lower Phase | 74.10 ± 3.28 |
| 3  | 8       | 2     | 0     | 5    | 5   | 0.89     | 14 ± 3| Lower Phase | 73.77 ± 3.71 |
| 4  | 7       | 3     | 0     | 5    | 5   | 0.82     | 19 ± 3| Lower Phase | 72.13 ± 2.81 |
| 5  | 6       | 4     | 0     | 5    | 5   | 0.77     | 10 ± 2| Lower Phase | 73.77 ± 2.39 |
| 6  | 5       | 5     | 0     | 5    | 5   | 0.75     | 26 ± 3| Lower Phase | 71.48 ± 2.91 |
| 7  | 4       | 4     | 0     | 5    | 5   | 0.80     | 28 ± 4| Lower Phase | 70.16 ± 2.27 |
| 8  | 3       | 3     | 0     | 5    | 5   | 0.85     | 30 ± 3| Lower Phase | 63.93 ± 2.70 |
| 9  | 2       | 2     | 0     | 5    | 5   | 0.94     | 30 ± 4| Lower Phase | 63.91 ± 3.19 |
| 10 | 1       | 1     | 0     | 5    | 5   | 0.82     | 10 ± 2| Lower Phase | 64.10 ± 1.81 |
| 11 | 0       | 5     | 0     | 5    | 5   | 0.89     | 8 ± 1 | Lower Phase | 69.18 ± 2.80 |
| 12 | 0       | 4     | 1     | 0    | 5   | 1.00     | 20 ± 3| Lower Phase | 67.38 ± 2.29 |
| 13 | 0       | 3     | 2     | 0    | 5   | 1.11     | 14 ± 2| Lower Phase | 67.87 ± 2.70 |
| 14 | 0       | 2     | 3     | 0    | 5   | 1.20     | 11 ± 2| Lower Phase | 67.21 ± 1.90 |
| 15 | 0       | 1     | 4     | 0    | 5   | 1.30     | 14 ± 3| Lower Phase | 66.89 ± 2.16 |
| 16 | 0       | 0     | 5     | 0    | 5   | 1.27     | 17 ± 2| Lower Phase | 66.82 ± 2.82 |

Experimental conditions: rotation speed, 850 rpm; mode, head-to-tail; flow rate, 3 mL min⁻¹; separation temperature, 30 °C. VR: volume ratio; ST: setting time; MP: mobile phase; RR: retention rate.

Table 2. The retention rate of GUESS-based n-hexane–ethylacetate–methanol–water solvent system.

| No | nC₆H₁₄ | EtOAc | MeOH | H₂O | VR (U/L) | ST (s) | MP      | RR (%)  |
|----|---------|-------|------|-----|----------|--------|---------|---------|
| 1  | 9       | 1     | 9    | 1   | 0.73     | 7      | Lower Phase | 74.75 ± 2.11 |
| 2  | 8       | 2     | 8    | 2   | 0.72     | 9      | Lower Phase | 73.77 ± 1.82 |
| 3  | 7       | 3     | 7    | 3   | 0.69     | 10     | Lower Phase | 71.15 ± 2.59 |
| 4  | 7       | 3     | 6    | 4   | 0.75     | 7      | Lower Phase | 73.44 ± 1.97 |
| 5  | 6       | 4     | 6    | 4   | 0.68     | 12     | Lower Phase | 73.61 ± 2.77 |
| 6  | 5       | 5     | 5    | 5   | 0.82     | 19     | Lower Phase | 72.13 ± 3.10 |
| 7  | 5       | 5     | 5    | 5   | 0.77     | 10     | Lower Phase | 73.77 ± 2.74 |
| 8  | 4       | 6     | 5    | 5   | 0.69     | 21     | Lower Phase | 67.54 ± 2.27 |
| 9  | 4       | 6     | 5    | 5   | 0.66     | 35     | Lower Phase | 61.64 ± 2.90 |
| 10 | 3       | 7     | 5    | 5   | 0.82     | 28     | Lower Phase | 64.92 ± 1.84 |
| 11 | 4       | 6     | 4    | 6   | 0.83     | 42     | Lower Phase | 62.62 ± 2.05 |
| 12 | 3       | 7     | 4    | 6   | 0.90     | 39     | Lower Phase | 63.28 ± 2.43 |
| 13 | 2       | 8     | 2    | 8   | 0.94     | 53     | Lower Phase | 60.66 ± 2.95 |
| 14 | 1       | 9     | 1    | 9   | 0.96     | 33     | Lower Phase | 64.92 ± 2.00 |
| 15 | 0       | 10    | 0    | 10  | 0.89     | 8      | Lower Phase | 69.18 ± 1.79 |

Experimental conditions: rotation speed, 850 rpm; mode, head-to-tail; flow rate, 3 mL min⁻¹; separation temperature, 30 °C. VR: volume ratio; ST: setting time; MP: mobile phase; RR: retention rate. Note: GUESS is a practical approach for the prediction of CCC distribution constants, K values, by standard thin layer chromatography [9].

The overall settling time of chloroform–methanol–water solvent systems is shorter than that of n-hexane–ethylacetate–methanol–water systems because the density difference of the two liquid phases of the former systems is larger than that of the latter solvent systems. Thus, a relatively high retention rate can be obtained using chloroform–methanol–water solvent systems, ranging from 70.16% to 76.39% (Table 3).
Table 3. The retention rate of chloroform–methanol–water solvent system.

| No | CHCl₃ | MeOH | H₂O | VR (L/U) | ST (s) | MP       | RR (%) |
|----|-------|------|-----|----------|--------|----------|--------|
| 1  | 10    | 0    | 10  | 0.98     | 6      | Lower Phase | 72.13 ± 1.66 |
| 2  | 10    | 1    | 9   | 1.00     | 8      | Lower Phase | 76.39 ± 2.00 |
| 3  | 10    | 2    | 8   | 1.04     | 12     | Lower Phase | 74.43 ± 2.38 |
| 4  | 10    | 3    | 7   | 1.06     | 14     | Lower Phase | 71.15 ± 1.97 |
| 5  | 10    | 4    | 6   | 1.09     | 10     | Lower Phase | 74.45 ± 2.70 |
| 6  | 10    | 5    | 5   | 1.15     | 11     | Lower Phase | 74.10 ± 2.50 |
| 7  | 10    | 6    | 4   | 1.27     | 10     | Lower Phase | 74.43 ± 2.82 |
| 8  | 10    | 7    | 3   | 1.86     | 15     | Lower Phase | 70.16 ± 1.92 |
| 9  | 10    | 8    | 2   | -        | -      | -         | -      |

Experimental conditions: rotation speed, 850 rpm; mode, head-to-tail; flow rate, 3 mL min⁻¹; separation temperature, 30 °C. VR: volume ratio; ST: setting time; MP: mobile phase; RR: retention rate.

Normally, n-heptane–n-butanol–acetonitrile–water solvent systems are not commonly used in HSCCC (Table 4), although an ideal retention rate can still be achieved at the ratio of 4:2.6:2.4:1 v/v/v/v (71.15%) and with n-heptane–acetonitrile at the ratio of 5:5 v/v (75.08%). Interestingly, the settling time of n-heptane–n-butanol–acetonitrile at the ratio of 5:2:3 v/v/v is 27 s, but the retention of the stationary phase is zero at a rotation rate of 850 rpm. Additionally, the settling time of both n-heptane–n-butanol–acetonitrile–water at the ratio of 4:3.2:1.8:2 v/v/v/v and n-butanol–water at 5:5 v/v was 16 s, however, the retention ratio of the former is reduced almost 50% of that of the latter.

Table 4. The retention rate of n-heptane–n-butanol–acetonitrile–water solvent system.

| No | nC₇H₁₆ | nBuOH | ACN | H₂O | VR(U/L) | ST(s) | MP      | RR(%)   |
|----|--------|-------|-----|-----|---------|-------|---------|---------|
| 1  | 5      | 0     | 5   | 0   | 0.82    | 5     | Lower Phase | 75.08 ± 2.71 |
| 2  | 5      | 2     | 3   | 0   | 0.79    | 27    | Lower Phase | 0       |
| 3  | 4      | 2.6   | 2.4 | 1   | 0.61    | 7     | Lower Phase | 71.15 ± 2.30 |
| 4  | 4      | 3.2   | 1.8 | 2   | 0.59    | 16    | Lower Phase | 33.44 ± 2.54 |
| 5  | 2      | 3.8   | 1.2 | 4   | 1.63    | 46    | Lower Phase | 52.79 ± 2.39 |
| 6  | 1      | 4.4   | 0.6 | 4   | 1.67    | 41    | Lower Phase | 63.61 ± 3.01 |
| 7  | 0      | 5     | 0   | 5   | 1.27    | 16    | Lower Phase | 66.89 ± 2.88 |

Experimental conditions: rotation speed, 850 rpm; mode, head-to-tail; flow rate, 3 mL min⁻¹; separation temperature, 30 °C. VR: volume ratio; ST: setting time; MP: mobile phase; RR: retention rate.

To explore the relationship between settling time and the retention of the stationary phase, the linear correlation coefficient of tested solvent systems was investigated. As shown in Figure 2, the GUESS-based n-hexane–ethylacetate–methanol–water solvent systems showed the highest correlation coefficient (r = -0.91, n = 16), followed by classic n-hexane–ethylacetate–methanol–water solvent systems (r = -0.60, n = 16), chloroform–methanol–water solvent systems (r = -0.54, n = 8) and n-heptane–n-butanol–acetonitrile–water solvent systems (r = -0.26, n = 7). These results indicated that the settling time is not always inversely proportional to the retention of the stationary phase. It is of great importance to test the retention of the stationary phase of solvent systems using the HSCCC apparatus.
The retention of the stationary phase of each solvent system is more accurate for HSCCC solvent system selection, since settling time is not always inversely proportional to the retention of the stationary phase.

As early as 1993, Berthod and Schmidt studied the stationary phase retention of water–organic solvent systems in CCC [13]. However, the investigated solvent systems were composed of two pure solvents only, which were seldom applied in HSCCC. This study investigated the retention of the stationary phase of classical biphasic solvent systems using a TBE300C HSCCC apparatus. Our results revealed that compared with settling time, the retention of the stationary phase of each solvent system is more accurate for HSCCC solvent system selection, since settling time is not always inversely proportional to the retention of the stationary phase.

Among the tested solvent systems, the n-hexane–ethylacetate–methanol–water solvent systems showed the highest correlation coefficient of settling time and the retention of the stationary phase (r = −0.91, n = 16). Nevertheless, the n-heptane–n-butanol–acetonitrile–water solvent systems showed the lowest correlation coefficient (r = −0.26, n = 7). Intriguingly, we found that the settling time of n-heptane–n-butanol–acetonitrile at the ratio of 5:2:3 is 27 s, but the retention of the stationary phase is zero at a rotation rate of 850 rpm. The reason for this phenomenon is as an anhydrous solvent, the violent pulsation may form a severe emulsion in the column which leads to the loss of the stationary phase. In addition, the density difference between the two liquid phases of this solvent system is too small, which leads to the loss of the stationary phase [14].

Additionally, it is noticed that after sample separation, the retention of the stationary phase of the solvent system is smaller than that value obtained after the hydrodynamic equilibrium. For example, in this study, the settling time of n-hexane–ethylacetate–methanol–water solvent system (A, n = 16), classic n-hexane–ethylacetate–methanol–water solvent systems (B, n = 16), chloroform–methanol–water solvent systems (C, n = 8) and n-heptane–n-butanol–acetonitrile–water solvent systems (D, n = 7).

**4. Discussion**

Figure 2. The linear correlation analysis of settling time and the retention of the stationary phase of GUESS–based n-hexane–ethylacetate–methanol–water solvent systems (A, n = 16), classic n-hexane–ethylacetate–methanol–water solvent systems (B, n = 16), chloroform–methanol–water solvent systems (C, n = 8) and n-heptane–n-butanol–acetonitrile–water solvent systems (D, n = 7).
equilibrium is established in the HSCCC column. This is because sample loading and mobile phase elution could result in some stationary phase reductions in the separation procedure. For example, in this study, the retention of the stationary phase of n-hexane–ethylacetate–methanol–water (1:1:1:1, v/v/v/v) was 71.48% after hydrodynamic equilibrium was established, while after myricanol separation, the value was only slightly reduced to 67.21%. Importantly, although various instrument types and different experiment conditions (temperature, rotation speed and flow rates of mobile phase) were applied, the retention of the stationary phase obtained in our study is quite similar to previous works. For instance, the retention of the stationary phase of n-hexane–ethylacetate–methanol–water (1:1:1:1, 4:6:4:6 and 3:5:3:5, v/v/v/v) in a recent study was 75%, 70% and 65%, respectively [15], and our data for these solvent systems are 71.48%, 64.92% and 63.93%. The retention of the stationary phase of n-butanol–ethylacetate–water (2:3:5, v/v/v) is 70% in Xuan et al.’s work [16] and 67.87% in our study. Taken together, the retention of the stationary phase of various solvent systems provided in this study would help select solvent systems for HSCCC application, because when changing the ratio of certain reagents in classic solvent systems, one could finally obtain an ideal one with proper retention of the stationary phase and partition coefficient of target compounds.

5. Conclusions

This study presented the retention of the stationary phase of several classical biphasic solvent systems using a TBE300C HSCCC apparatus. The results revealed that settling time is not always inversely proportional to the retention of the stationary phase of HSCCC. The values of the retention of the stationary phase of various solvent systems will be helpful for HSCCC solvent system selection and application of this technique.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9110357/s1, Figure S1: The 1H and 13C NMR spectroscopic data of myricanol.

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