

Purification of Flavonoids from Mulberry Leaves via High-Speed Counter-Current Chromatography

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Keywords: separation, high-performance liquid chromatography, high-speed counter-current chromatography, flavonoids, mulberry leaf

Abstract:

In order to obtain high-purity flavonoid products, the extracts from mulberry leaves were separated and purified via high-speed counter-current chromatography (HSCCC). Moreover, the product was detected via high-performance liquid chromatography (HPLC). The characteristic absorption wavelength of the rutin standard for HSCCC detection and HPLC analysis at 257 nm was tested by ultraviolet scanning analysis. The effect of solvent systems and mobile phase flow rate on the separation efficiency were then researched. Finally, the solvent system of V(ethyl acetate):V(n-butanol):V(water) = 4:1:5 was selected as the operating system for HSCCC. This work theoretically analyzed the impact of the molecular structure and polarity of flavonoids on the choice of solvent systems. The results showed that the mobile phase flow rate had a great influence on the separation efficiency. Furthermore, the separation efficiency increased as the mobile phase flow rate decreased. When the mobile phase flow rate was 5 mL/min, the peak time for flavonoids was 140 min, the retention of the stationary phase was 56.4%, and the purity of the product reached 93.8%. The results of this study greatly improved the purity of flavonoids in mulberry leaf and provided a strong support for the separation and purification of mulberry leaf extract.

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Article

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Abstract: In order to obtain high-purity flavonoid products, the extracts from mulberry leaves were separated and purified via high-speed counter-current chromatography (HSCCC). Moreover, the product was detected via high-performance liquid chromatography (HPLC). The characteristic absorption wavelength of the rutin standard for HSCCC detection and HPLC analysis at 257 nm was tested by ultraviolet scanning analysis. The effect of solvent systems and mobile phase flow rate on the separation efficiency were then researched. Finally, the solvent system of V(ethyl acetate):V(n-butanol):V(water) = 4:1:5 was selected as the operating system for HSCCC. This work theoretically analyzed the impact of the molecular structure and polarity of flavonoids on the choice of solvent systems. The results showed that the mobile phase flow rate had a great influence on the separation efficiency. Furthermore, the separation efficiency increased as the mobile phase flow rate decreased. When the mobile phase flow rate was 5 mL/min, the peak time for flavonoids was 140 min, the retention of the stationary phase was 56.4%, and the purity of the product reached 93.8%. The results of this study greatly improved the purity of flavonoids in mulberry leaf and provided a strong support for the separation and purification of mulberry leaf extract.

Keywords: mulberry leaf; flavonoids; high-speed counter-current chromatography; high-performance liquid chromatography; separation

1. Introduction

Mulberry leaves are rich in active compounds, as they contain a variety of vitamins, amino acids, polysaccharides, flavonoids, alkaloids, and a large number of essential microelements [1,2]. These plants are cultivated in most areas of China and have a very high medicinal value, demonstrating positive effects on clearing the liver and eyes, lowering blood pressure, correcting blood lipid and blood sugar levels, as well as countering hypertension and virus infections [3–5]. Studies assessing the active components of mulberry leaves used in traditional Chinese medicine have been extensively reported, and the antihypertensive, hypolipidemic, and antioxidative effects of flavonoids have received much attention among researchers and manufacturers [6,7].

The flavonoids present in mulberry leaves are a complex mixture containing a variety of compounds including rutin, quercetin, isolicorices, anthocyanins, and other flavonoids [8]. At present, the separation methods used to isolate flavonoids mainly include macroporous resin adsorption, silica gel column chromatography, ion exchange resin, and cellulose layer column methods [9–12]. Macroporous resin adsorption has the advantages of a large specific surface area, high physical and chemical stability, a large adsorption capacity, and recyclability. Silica gel column chromatography has an advantage as the stationary phase is stable and does not easily decompose. Ion exchange resins have the advantages of a high exchange capacity, low regenerant consumption, and long service life.

For the cellulose layer column method, the matrix material is not easily broken and has high adhesive strength. Additionally, the components are inexpensive and easy to obtain. However, regardless of the method used, the refining processes involved with these methods are long and cumbersome, and obtaining high-purity flavonoid products is difficult.

Alternatively, high-speed counter-current chromatography (HSCCC) has proven to be a versatile and dynamic separation method that is faster and more efficient than most traditional methods. Unlike traditional solid–liquid column chromatography, HSCCC is a continuous and efficient liquid–liquid partition chromatographic separation technology, as both the stationary and mobile phases are solutions. The hydrodynamic equilibrium of the two-phase solvent system is established in a high-speed rotating spiral tube. Since solid supports are not required, sample loss and denaturation caused by irreversible adsorption are avoided, and the purity of the product is improved by the continuous eluting process. As such, HSCCC has been widely used in the separation and preparation of natural active ingredients from plants in recent years [13–15]. For example, the purity of astaxanthin isolated from *Haematococcus pluvialis* can reach as high as 99% when purified via HSCCC, but the content of astaxanthin extracted by solvent extraction is only about 30% [16,17]. Additionally, the preparation and purification of oleuropein from olive leaves by HSCCC can reach a purity of more than 90%, whereas purification via dextran gel is only 41.5%, and the use of a macroporous resin can only generate a mass fraction of oleuropein in solids of 21.6% [18–20]. The anthocyanin purity of blueberry anthocyanin can be as high as 32.0% when purified via macroporous resin, but the content of anthocyanin from red grape skins achieved over 91% purity when isolated via HSCCC [21,22].

HSCCC has been successfully used to obtain high-purity coumarins, fatty acids, and other active components from mulberry leaves [23]. In this current study, a method for the purification of flavonoids from mulberry leaves via HSCCC was established for the first time. The characteristic absorption wavelength for HSCCC detection and HPLC (high-performance liquid chromatography) analysis was determined by way of UV scanning analysis of a rutin standard. The solvent systems and the effect of the mobile phase velocity on the separation efficiency were studied and analyzed, and the optimal solvent system and process conditions were determined. The results showed that the purity of flavonoids, as determined by HPLC, from mulberry leaves was greatly improved. The advantages of the HSCCC method include high purity, simple operation, large processing capacity, and short separation time. Therefore, this method provides a new approach for the purification of flavonoids from mulberry leaves.

2. Materials and Methods

2.1. Materials

Mulberry leaves were grown in Sichuan and conserved in a dry and ventilated condition after being smashed by a portable pulverizer. Rutin standard with purity $\geq 98\%$ came from Aladdin Reagent Co., Ltd., Shanghai, China. Analytical ethanol, ethyl acetate, n-butanol, and phosphoric acid were produced in Sinopharm Chemical Reagent Co., Ltd., Wuhan, China. Chromatographic methanol was obtained from Tianjin Xiehe Kunpeng Chromatography Technology Co., Ltd., Tianjin, China.

2.2. Extraction of Flavonoids from Mulberry Leaves

The mulberry leaves were crushed using a portable pulverizer (HK-04A, Guangzhou Xulang Machinery Equipment Co., Ltd., Guangzhou, China). A total of 5.0 g of dried mulberry leaf powder was added to a 60% ethanol solution, and ultrasonic extraction was carried out for 15 min at an extraction temperature of 60 °C and a power of 325 W. The mulberry leaf extract was collected after vacuum filtration and concentrated using a rotary evaporator (SY-2000, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) to obtain a brown viscous material that served as a crude extract for the flavonoids.

2.3. Separation and Purification of Mulberry Leaf Extracts

The HSCCC was equipped with an ultraviolet-visible spectrophotometer (UV-1800PC, Shanghai Meipuda Instrument Co., Ltd., Shanghai, China), which was used to determine the collected time of the target product by monitoring the chromatogram at a specific wavelength. After the solvent system was prepared, the two-phase solution was separately transferred into two glass vials. The upper phase was used as a stationary phase, and the lower phase was used as a mobile phase. The stationary phase was pumped into the spiral column of the HSCCC (TBE-300C, Shanghai Tongtian Biotechnology Co., Ltd., Shanghai, China) at a flow rate of 30 mL/min. When the stationary phase was filled from the liquid outlet, the pump was stopped. The main power was then turned on, the host speed was set to 800 rpm/min, and the FWD was forwarded. When the stationary phase and the mobile phase had reached equilibrium in the HSCCC column, the prepared mulberry leaf crude extract sample was injected into the HSCCC instrument. The chromatographic workstation was then run with a UV detection wavelength of 257 nm. Subsequently, the separated and purified components were collected according to the chromatogram.

2.4. Analysis by HPLC

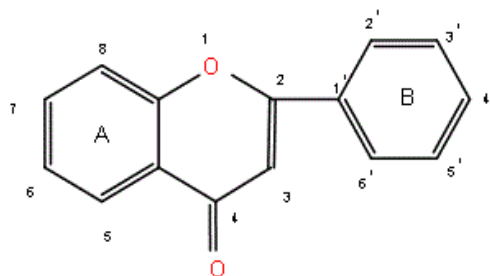
High-performance liquid chromatography (HPLC) (Dionexu-3000, Dai'an China Co., Ltd., Beijing, China) was used to analyze the product contents [24]. A total of 0.05 g of the refined product was dissolved in ethanol in a 100-mL volumetric flask. The mobile phase was V(chromatographic methanol):V(0.5% aqueous phosphoric acid) = 50:50, the column temperature was 30 °C, the flow rate was 1.0 mL/min, and the injection volume was 30 µL. Finally, the peak time and peak area were detected at the characteristic wavelength of 257 nm.

3. Results

3.1. Separation and Purification via HSCCC

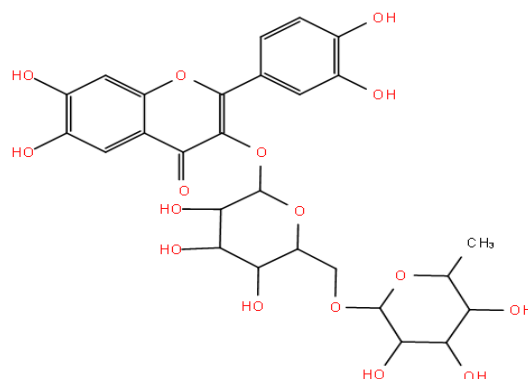
3.1.1. Determination of the Characteristic Absorption Wavelength

Because flavonoids are a mixture, it is difficult to analyze them properly. As such, flavonoid monomers with the same parent nucleus structure must be selected for such experiments. Rutin has the same parent nucleus structure as flavonoids, as shown in Figure 1. When separating and purifying flavonoids from mulberry leaf extracts via HPLC, rutin is typically used as the standard. According to the different functional groups of different substances, the characteristic absorption peaks under UV irradiation were different. The rutin standard was scanned directly using a UV-visible spectrophotometer at full wavelength.



(a) Basic nucleus of flavonoids.

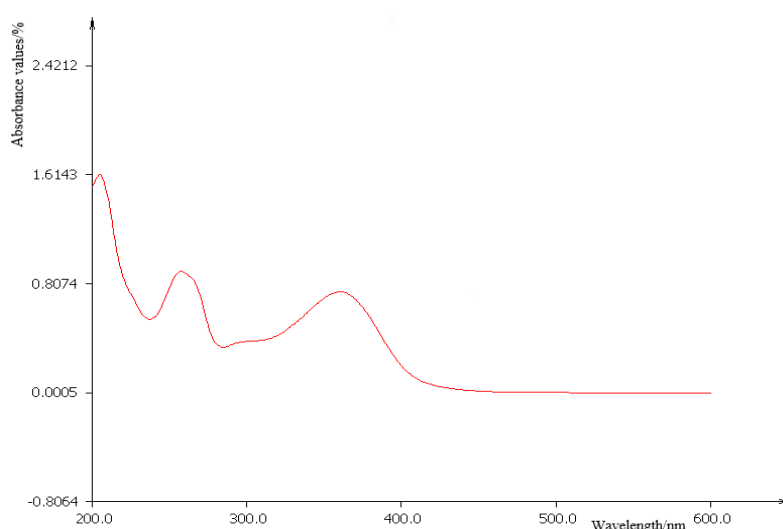
Figure 1. Cont.



(b) Molecular structure of rutin.

Figure 1. The basic nucleus of flavonoids and the molecular structure of rutin.

The analysis of the scan of the rutin standard is shown in Figure 2. The characteristic absorption peaks of rutin were determined to be 205 nm, 257 nm, and 306 nm. Comparing these three wavelengths, the absorbance decreased in turn, and the maximum absorption peak appeared at 205 nm. However, the weak stability at this wavelength means that it cannot be used as the analysis wavelength of the rutin standard. As such, 257 nm was selected as the characteristic absorption wavelength for our analysis.

**Figure 2.** The ultraviolet spectrum of the rutin standard.

3.1.2. Separation Performance of the Solvent System

A suitable solvent system is an important factor affecting the separation efficiency of HSCCC. Ethyl acetate, n-butanol, water and ethyl acetate, ethanol, water were selected as the basic solvent systems, and each basic system was formulated into four solutions of different compositions. The rutin standard was used as the separation medium to examine the solvent system. The preliminary screenings of the solvents were based on two parameters: the stratification time (t/s) and the partition coefficient (k).

$$K = C_U/C_L \quad (1)$$

where C_U is the concentration of the rutin standard in the upper liquid phase when the solvent system reaches equilibrium, $\mu\text{g/mL}$.

C_L is the concentration of the rutin standard in the lower liquid phase when the solvent system reaches equilibrium, $\mu\text{g/mL}$.

As shown in Table 1, the results indicated that the partition coefficients of solvent systems 1 and 4 were 0.60 and 0.52, respectively, which resulted in the peak time in the HSCCC to be too fast and the separation between the peaks to be poor. The component peaks and impurity peaks of rutin are not easy to separate, and this can affect the separation efficiency. The distribution coefficient of system 7 was 2.09; this larger distribution coefficient made the peak time too long and the peak shape relatively wide, resulting in a low separation effect, which decreased the purity of product. However, if the content of rutin in the fixed phase was too high, which is not conducive to subsequent separation. The stratification time of system 3 was long, and such an extended stratification period made the solvent system a poor choice for HSCCC, as it is prone to miscibility and can affect the retention of the stationary phase. Importantly, if the peak time is too long, the peak shape will be wide. Therefore, the solvent systems 2, 5, 6, and 8 were selected for the further optimization of the HSCCC separation.

Table 1. Separation performance of solvent systems.

Number	Solvent Ratio Volume	Separation Time (s)	Partition Coefficient (K)	Polarity Parameter of the Ideal Solvent System (f)
1	ethyl acetate/ethanol/water: 4/1/5	22.0	0.60	7.29
2	ethyl acetate/ethanol/water: 5/2/5	24.7	1.03	6.80
3	ethyl acetate/ethanol/water: 3/1/5	31.7	0.80	7.61
4	ethyl acetate/ethanol/water: 6/1/5	16.2	0.52	6.81
5	ethyl acetate/n-butanol/water: 4/1/5	19.4	1.08	7.25
6	ethyl acetate/n-butanol/water: 5/2/5	21.4	1.33	6.73
7	ethyl acetate/n-butanol/water: 3/1/5	27.6	2.09	7.54
8	ethyl acetate/n-butanol/water: 6/1/5	25.2	1.37	6.76

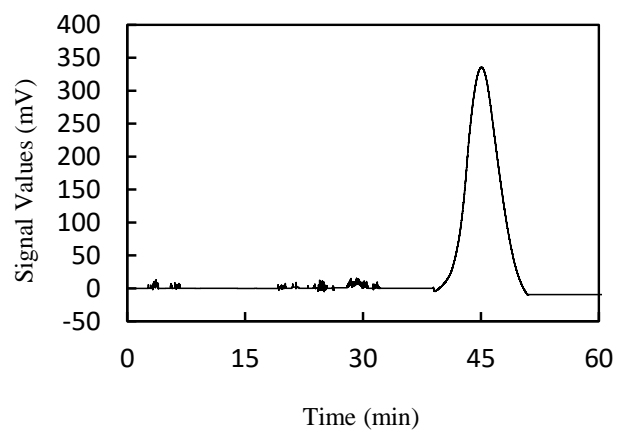
3.1.3. HSCCC of the Rutin Standard and Solvent Selection

As mentioned earlier, rutin was used as the separation medium. Under the same separation conditions, the peak time of rutin and the retention rate of the stationary phase were investigated under HSCCC utilizing the four solvent systems. The operating conditions were: a stationary phase flow rate of 30 mL/min, a mobile phase flow rate of 8 mL/min, a host rotation speed of 800 rpm/min, a water bath temperature of 25 °C, and an ultraviolet detection wavelength of 257 nm.

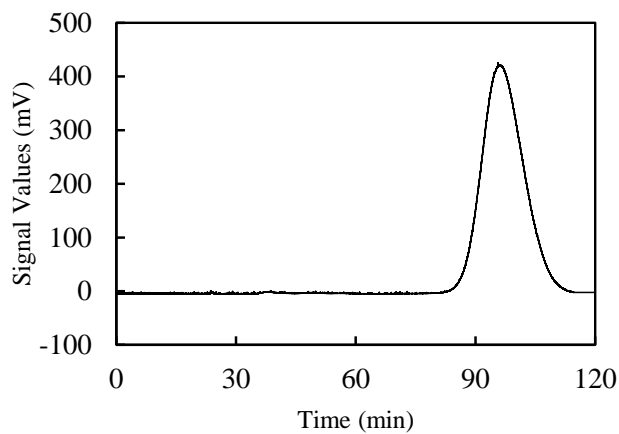
The results presented in Figure 3 and Table 2 indicate that the stationary phase retentions of solvents 8 and 2 were 5.6% and 27.8%, respectively. Such retentions of the stationary phase were too low, as the mobile phase took more stationary phases away from the HSCCC, which affected the separation efficiency and prevented the identification of the chromatogram peaks of rutin. Among the four solvent systems, the longest peak time was 140 min for solvent 6, with a retention rate of the stationary phase of 36.8%. The peak time of solvent 5 was 98 min, with a retention rate of the stationary phase of 50.8%, which satisfied the separation requirement of HSCCC. The other solvents had stationary phase retention rates of less than 40%.

Table 2. Separation effect of different solvent systems on the rutin standard.

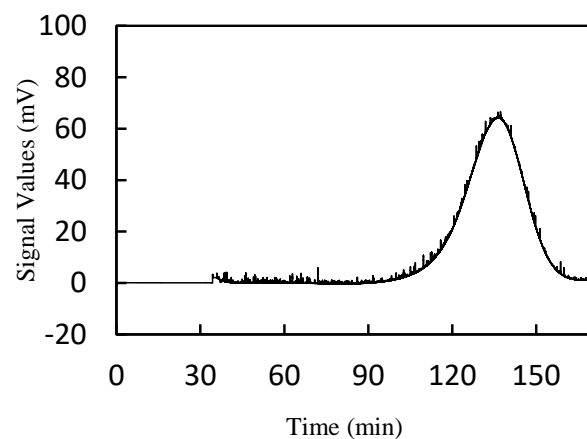
Solvent Systems	Peak Time (min)	Stationary Phase Retention (%)
Solvent 2	45	27.8
Solvent 5	98	50.8
Solvent 6	140	36.8
Solvent 8	-	5.6



(a)



(b)



(c)

Figure 3. High-speed counter-current chromatography (HSCCC) chart of the rutin standard in different solvent systems. (a) Solvent system 2; (b) solvent system 5; (c) solvent system 6.

Considering the stratification time and partition coefficient, as well as the retention rate and peak time of the stationary phase, it was decided that solvent 5 was the best solvent system for the separation of flavonoids from mulberry leaves via HSCCC.

There are many factors that can influence the choice of solvent systems. The main factor is the molecular polarity of the compound to be separated. Flavonoids have a certain polarity, as they present as a series of compounds in which two benzene rings (A-ring and B-ring) having a phenolic hydroxyl group are linked by a central three-carbon atom, as shown in Figure 1a, and the basic mother nucleus is a 2-phenylchromanone. Figure 1b shows the molecular structure of a typical flavonoid rutin. The parent structure of rutin is a 2-phenylchromanone, which is a cross-conjugated system. The carbonyl group in this structure is conjugated to two benzene rings with a phenolic hydroxyl group. The ring is conjugated and becomes a strong electron-withdrawing group under the superposition of the electron-withdrawing induction effect and the electron-withdrawing conjugation effect. Relatively speaking, the polarity of the ether bond is weak, and there is a plurality of strong electron-donating hydroxyl groups in the molecule, but their distribution is divergent, and their effect is relatively weak. Therefore, the polarity in the molecule is mainly due to the action of the carbonyl group. The structure of the hydroxyl group also gives it a certain solubility in water.

Because rutin has a certain polarity and hydrophilicity, according to the principle of similar compatibility, the solvent system should also have a certain polarity. When water, a polar molecule, is chosen as one of the basic components of the solvent system, ethyl acetate and alcohol can be used to adjust the polarity of the solvent system. Ignoring the influence of mixing between the different components in regard to polarity, and assuming that the solvent systems are ideal systems, the polarity parameters of the solvent systems can simply be linearized [25].

$$f = \sum_{i=1}^3 \alpha_i f_i \quad (2)$$

In the formula,

- f is the polar parameter of the ideal solvent system;
- α_i is the volume fraction of component i ;
- f_i is the polar parameter of component i .

The polar parameters of ethyl acetate, n-butanol, water, and ethanol were 4.4, 3.9, 10.2, and 4.3, respectively. The parameters of the components in the solvent system were water > ethyl acetate > ethanol > n-butanol, and rutin had a medium polarity. Therefore, the solvent scheme used in this study was based on the volume fraction of water of 50%, with the other water contents being 42% and 56%, respectively. The polar calculation results of the ideal solvent systems are shown in Table 1. The results indicated that solvent 5 was the optimal solvent system, as the polarity of solvent systems 1, 3, and 7 were too large compared to the isolated flavonoid compound, and those of the solvent systems 2, 4, 6, and 8 were too small.

The polarity of the flavonoids was analyzed, and a reversed-phase column was used for HSCCC. The upper phase of the solvent system was the stationary phase, the lower phase was the mobile phase, and the polarity of the stationary phase was smaller than that of the mobile phase.

3.1.4. Influence of the Mobile Phase Velocity on the Separation Efficiency

On the basis of the aforementioned research, the crude extracts of mulberry leaves were separated and purified by HSCCC using solvent system 5. The stationary phase flow rate was 30 mL/min, the host speed was 800 rpm/min, the water bath temperature was 25 °C, and the UV detector was set at 257 nm. By changing the flow rate of the mobile phase, its impact on the separation was investigated.

Under the same operating conditions, the chromatogram of the rutin standard and the chromatogram of the mulberry extract, as shown in Figures 3b and 4b, were compared. The peak of the mulberry extract showed that the second peak contained flavonoids. Because the crude extracts of mulberry leaves are a mixture with two peaks, there is a certain delay in the peak time of the corresponding product peak.

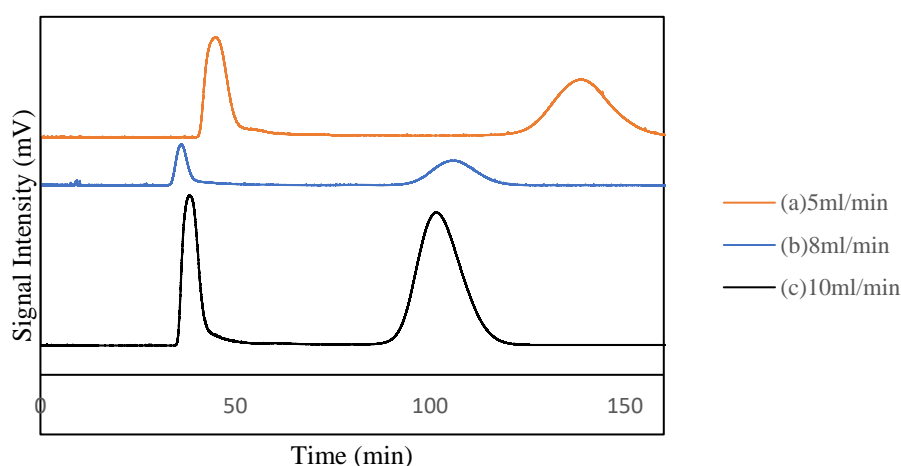


Figure 4. HSCCC chart of mulberry leaf crude extracts with different flow phase velocities of (a) 5 mL/min, (b) 8 mL/min, and (c) 10 mL/min.

The results presented in Figure 4 and Table 3 indicate that the mobile phase flow rate had a substantial influence on the separation effect. As the flow rate of the mobile phase was decreased, the peak time of the corresponding flavonoid increased. The longer the separation time, the better the separation effect, and the higher the retention rate of the stationary phase [26]. When the mobile phase flow rate was 5 mL/min, the peak time of the flavonoid was 140 min, the retention of the stationary phase was 56.4%, and the purity of the product was as high as 93.8%.

Table 3. Effect of mobile phase flow rate on the separation effect.

Mobile Phase Flow Rate (mL/min)	Peak Time (min)	Stationary Phase Retention (%)	Product Purity (%)
5	140	56.4	93.8
8	100	50.8	89.2
10	85	45.0	81.2

The two phases of the HSCCC were all solvents. The stationary phase was first injected into the spiral tube to stabilize, and the mobile phase was then injected to establish a special unidirectional hydrodynamic equilibrium between the two phases in the spiral tube. Therefore, when the flow velocity of the mobile phase changed, the dynamic balance between the two phases also changed [27], and this resulted in a change in the retention rate of the stationary phase in the spiral tube. Moreover, the dissolution and elution times of the sample in the two phases changed and the product purity was also affected. The smaller the mobile phase flow rate was, the greater the retention rate of the separated compound in the stationary phase. The more times the sample was dissolved and eluted in the two phases, the higher the product purity. However, the separation time also greatly increased.

3.2. Analysis by HPLC

Under the same chromatographic operating conditions, the rutin and the flavonoids isolated and purified by HSCCC were assessed by HPLC, and their purity was analyzed using the area normalization method.

Figure 5 presents the chromatogram of the rutin standard and flavonoids. It can be seen that the peak shapes of the two figures are close, and the retention times are consistent, with both being 5.9 min. The product collected by separating and purifying the crude mulberry leaf extracts via HSCCC contained flavonoid compounds. The purity of the product detected by HPLC is shown in Table 3.

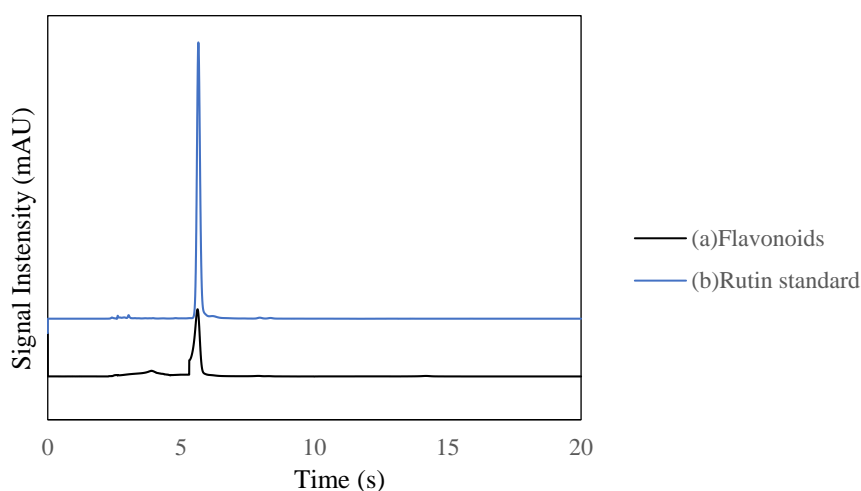


Figure 5. The high-performance liquid chromatography of (a) flavonoids and (b) the rutin standard.

4. Conclusions

Mulberry leaf extracts were separated and purified via HSCCC, and the obtained flavonoid products were detected by HPLC. The UV scan of rutin was analyzed to determine the characteristic absorption wavelength for HSCCC detection and HPLC analysis at 257 nm. Considering the stratification time, partition coefficient, retention rate, and peak time of the stationary phase in HSCCC, we determined that the V(ethyl acetate):V(n-butanol):V(water) = 4:1:5 system was the best solvent system, with a stratification time of 19.4 s and a distribution coefficient of 1.08. The effects of the molecular structure and polarity of the flavonoids on the solvent systems were analyzed theoretically. The results showed that as the flow rate of the mobile phase was decreased, the longer the peak time of the flavonoids, the higher the retention rate of the stationary phase, and the better the separation effect. When the mobile phase flow rate was 5 mL/min, the peak time of the flavonoids was 140 min, the retention of the stationary phase was 56.4%, and the purity of the product reached 93.8%. These results indicate a greatly improved purity of flavonoids isolated from mulberry leaves and provide a material basis for the application of flavonoids from mulberry leaves. Moreover, this study offers strong supports for the separation and purification of flavonoids via HSCCC.

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