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Separation of two constituents from purple sweet potato by combination of silica gel column and high-speed counter-current chromatography

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ABSTRACT

It is known that the choice of solvent system for high speed counter-current chromatography separation is of utmost importance. In this study, a simple and rapid thin layer chromatograph coupling with fluorometric (TLC-F) method has been used to determine the partition coefficient of target compounds in HSCCC solvent system. Two components, 6,7-dimethoxycoumarin and 5-hydroxymethyl-2-furfural were successfully separated from purple sweet potato extracts by successive sample injection for the first time, using n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) as the solvent system. Additionally, statistical analysis showed that there was no significant difference in partition coefficient obtained by the TLC-F method and by HPLC, which demonstrated the usefulness of TLC-F method.

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1. Introduction

Purple sweet potato [*Ipomoea batatas* (L.) Lam] originated from Central America, is now widely grown in the world. As other plant foods, purple sweet potato (PSP) accumulates a great variety of secondary metabolites, including phenolic acid, anthocyanins and glycoalkaloids [1,2]. Its starchy storage roots is one of the most widely used foods throughout the world, so determining its secondary metabolites is important in order to better understand the intake of these substances by humans. Among all secondary metabolites, the anthocyanins have attracted a great deal of attention due to its various biological activities, such as scavenging free radicals, antioxidant, antitumor, anti-hyperglycemia effect and reducing the risk of coronary and heart diseases [3–5]. And anthocyanins have been separated from PSP by a number of chromatographic methods [6,7]. However, in both animals and humans, the efficiency of anthocyanins absorption is relatively poor due to their rapid absorption and elimination [8,9]. Additionally, epidemiological studies have not revealed protective effects of anthocyanin consumption on cancer risk in humans, and their antioxidant activity in humans remains questionable [10]. Previous studies have suggested that other constituents except anthocyanins in PSP also possess pharmacological properties [11,12]. Our previous research also showed anthocyanins are not the only bio-active component that possess α-glucosidase inhibitory activity [13]. So, it is necessary to separate and identify other bioactive compounds in PSP.

High speed counter-current chromatography (HSCCC) is a liquid–liquid partition chromatographic technique in which a solid support matrix is not used. In contrast to prep-high performance liquid chromatography (HPLC), HSCCC has less solvent consumption, simpler separation conditions, larger loading, and no sample adsorption. The choice of solvent system for HSCCC separation is of utmost importance and it is estimated that about 90% of the entire work in HSCCC is invested in solvent system selection [14]. However, the lack of a clear method for comparing the relative merits of the many possible biphasic solvent systems available to the scientist seems to be a major drawback in the employment of HSCCC [15]. In HSCCC, the value of the partition coefficient (*K*) of the solute of interest should ideally range between 0.5 and 1.0 [16]. *K* lower than 0.5 brings a loss of resolution, while *K* higher than 1.0 causes a long retention time and leads to diluted solutes. The *K* values have widely been assessed by measuring the absorbance of the solute

Abbreviations: HSCCC, high speed counter-current chromatography; TLC-F method, thin layer chromatograph coupling with fluorometric method; PSP, purple sweet potato; HPLC, high performance liquid chromatography; *K*, partition coefficient; UV, ultraviolet spectrophotometric.

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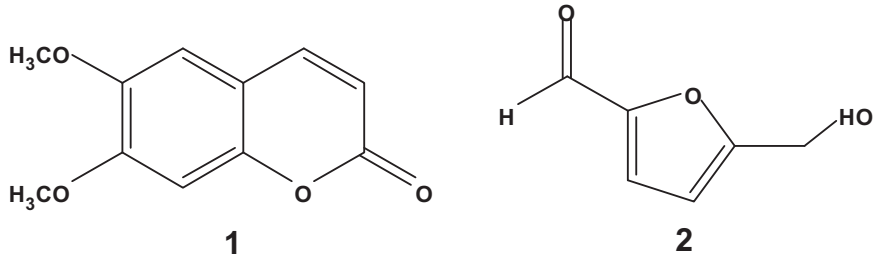

Fig. 1. Chemical structures of the 6,7-dimethoxycoumarin (1) and 5-hydroxymethyl-2-furfural (2).

in each of the two phases after partitioning in the equilibrated two-phase solvent system and dividing the solute concentration in the upper phase by that in the lower phase [17]. Thus, the HPLC and ultraviolet spectrophotometric (UV) method have been used to select solvent system for HSCCC separations. Although HPLC offers high sensitivity and separation efficiencies, it requires sophisticated equipment and select the eluent solvent is time-consuming and difficult. UV method obtains inaccurate results when the sample is a mixture of various components, which makes the usage of this method limited.

After partitioning the sample between the two solvent phases, the same volume of sample in two phases was separated by TLC and the separation results provided useful information about *K* values of target compounds [18]. However, macroscopic observations have failed to provide accurate results. In this study, a simple and precise thin layer chromatograph (TLC) coupling with fluorometric (TLC-F) method has been used to determine the partition coefficient of target compounds in HSCCC solvent systems. And a one step high speed counter-current chromatography method was developed for the separation and purification of 6,7-dimethoxycoumarin (component 1) and 5-hydroxymethyl-2-furfural (component 2) from purple sweet potato (Fig. 1).

2. Experimental

2.1. Materials and reagents

The purple sweet potato was provided by Chongqing Sweet potato Research Center. All solvents used for preparation of enriched extract and for HSCCC separation were of analytical grade (Kelong Chemical Reagent Factory, Chengdu, China). Methanol and acetonitrile used for HPLC were of chromatographic grade (Honeywell Burdick & Jackson, USA), and the water used was distilled water.

2.2. Apparatus

The HSCCC instrument employed in the present study was a TBE-300B high-speed counter-current chromatography (Tauto Biotech, Shanghai, China) with three multilayer coil separation columns connected in series (total volume = 300 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the values β ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. 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 on a LC-20AD system equipped with a diode 126 array detector (Shimadzu, Japan). ECOSIL-C18 column (4.6 mm × 250 mm, 5 μm particle size). Fluorometric measurements were made with a F-4500 spectrophotofluorometer (Hitachi, Tokyo, Japan).

2.3. Preparation of crude extract

The purple sweet potato root was chopped and dried in a drying oven at 50 °C for 10 h. After that, the PSP root was ground to pass through a 100 mesh screen. 1000 g of the powder was added to 10 L ethanol (acidified with 0.1% HCl) by ultrasonic extraction for 60 min at room temperature, then filtered. Filtrates were evaporated under the reduced pressure at 50 °C to afford the PSP residue. The residue was stored at 0 °C until use.

2.4. Pre-separation of PSP residue by silica gel column

The residue was dissolved in 1 L of distilled water and then extracted with petroleum ether and ethyl acetate, respectively. Each fraction was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The bioactive ethyl acetate fraction (10 g) was subjected to column chromatography using silica gel (100–200 mesh, 600 g) as adsorbent and elution was carried out with petroleum ether followed by gradually increasing polarity with acetone. Elution with petroleum ether: acetone mixture 95:5 and 90:10 afforded fractions A (with a gradient of 500 ml). After TLC analysis, this fraction was used for subsequent HSCCC isolation.

2.5. Evaluation of partition coefficient (K) value

The *K*-values were determined according to our previous method reported [19]. Briefly, a suitable amount of crude extract was dissolved in a 10 ml test tube to which 4 ml each of pre-equilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for several minutes to thoroughly equilibrate the sample with two phases. Then, equal volumes (1 μl) of the upper and lower phases were applied on a TLC plate, after separated by the TLC mobile phase, the spot of the target component were scraped from the plate and then extracted to measure the fluorescence intensity using fluorescence spectrophotometer. The *K* value can be obtained by divide the fluorescence intensity of sample in the upper phase by that in the lower phase. Besides, the *K* value was also measured by HPLC method according to Ito [16], to investigate is there any difference in *K* value measured by the two methods. Settling time of two-phase solvent system was expressed in terms of the time when the vigorously stirred mixture of upper and lower phase (1:1, v/v) forms a clear layer. The solvent volume of each phase was measured by a 10-ml graduated cylinder.

2.6. Thin-layer chromatography (TLC) and fluorometric analysis

TLC analysis was carried out on normal-phase silicagel plates (Qingdao Haiyang Chemical plant, Qingdao, China), and

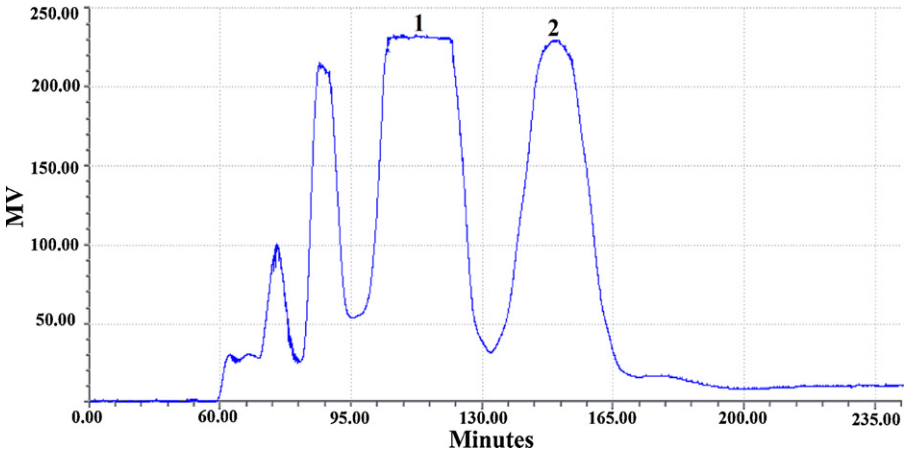

Fig. 2. HSCCC chromatogram of crude extracts from purple sweet potato. *Conditions*: solvent system: n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v); flow-rate: 2.0 ml/min; rotational speed: 810 rpm; detection wavelength: 254 nm; separation temperature: 25 °C; sample size: 120 mg dissolved in 10 ml upper phase; retention of the stationary phase: 55%. 1: 6,7-dimethoxycoumarin (collected during 98–123 min); 2: 5-hydroxymethyl-2-furfural (collected during 133–160 min). The other peaks were not identified.

chloroform–ethyl acetate (1:1, v/v) was used as the developing system. The analyte was detected by a UV detector at 254 nm. The spot of the target component (in upper phase and lower phase, respectively) was scraped in a 1.5 ml microcentrifuge tube and extracted with 1.2 ml methanol by sonication (100 kHz, 10 min). After that, the sample solvent was centrifuged at 15,000 rpm for 5 min, and 100 μl of the obtained supernatant was added to 2 ml methanol for fluorescent measurement. The fluorescent intensity of target compound in both upper phase and lower phase was measured on a F-4500 fluorescence spectrophotometer using an excitation wavelength of 340 nm and an emission wavelength of 427 nm (with 5 nm bandwidth).

2.7. HSCCC separation procedure

After thoroughly equilibrating the mixtures in a separatory funnel at room temperature, two phases were separated shortly before use. The multilayer coil column was first entirely filled with the upper phase, the lower phase was then pumped into the head end of the inlet column at a flow rate of 2.0 ml/min, while the apparatus was rotated at 810 rpm. After reaching hydrodynamic equilibrium, the sample solution (120 mg of the crude extract in 10 ml organic phase) was injected into the column through the sample loop. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and the chromatogram was recorded. Eluates were collected into test tubes at 2 min intervals per tube. When the separation was completed, the solvents in the column were pushed out to estimate the retention ratio of the solvent system. Analysis of HSCCC fractions was done by TLC and HPLC.

2.8. HPLC analysis and identification of compounds

The crude extracts and each peak fraction from HSCCC were analyzed by HPLC. The column used in HPLC analysis was an ECOSIL-C18 column. Methanol and water with the ratio of 50:50 were used as the mobile phase, the flow rate was 1.0 ml/min, and the effluent was monitored at 254 and 345 nm. HSCCC peak fractions were identified by comparing their ^{1}H and ^{13}C NMR spectroscopic data with literature values. ^{1}H and ^{13}C NMR spectroscopic data were obtained on a Bruker GPX 500 spectrometer at 300 MHz, respectively.

3. Results and discussion

3.1. K values in n-hexane–ethyl acetate–methanol–water system and HSCCC separation

The n-hexane–ethyl acetate–methanol–water solvent system family is considered to separate compounds of lipophilic to moderate polarity and the partition coefficients (*K* values) of the target compounds in this solvent system were determined. As shown in Table 1, the *K* value of two components in system 1 and 4 was very close, which may lead to low resolution of analytes. Besides, unacceptable volume ratio of two phases of the solvent system 1 and 4 would result in solvent wastage. System 2 afforded a suitable *K* value for separating components 1 and 2 but both were contaminated by impurities and caused long separation time. So, n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) was used for HSCCC separation and the HSCCC chromatogram is shown in Fig. 2. 120 mg of crude extract was purified using the solvent system 3 and then yielded 15.1 mg component 1 (eluted out between 98 and 123 min) and 28.7 mg component 2 (eluted out between 133 and 160 min) upon recrystallization. The retention of the stationary phase was 55.0%.

The choice of solvent system for HSCCC separations is the most important and difficult step before using this advanced technology. In this study, a thin layer chromatograph coupling with fluorometric (TLC-F) method was developed to determine the partition coefficient of target pounds in HSCCC solvent system. To estimate the accuracy of this method, the *K* value of component 1 in n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) was determined by both HPLC and the new TLC-F method. After partitioning the sample between the two solvent phases, a proper same volume of the two phases, was analyzed by HPLC (Fig. C in Appendix). The *K* value of 6,7-dimethoxycoumarin was determined by computing the peak areas of the corresponding peaks (Table 2). TLC separation results of PSP extracts are shown in Fig. 3. It can be seen that 6,7-dimethoxycoumarin was well separated from other components, and the R_f value was 0.42. The fluorescent intensity of 6,7-dimethoxycoumarin in upper phase and lower phase was measured by fluorescence spectrophotometer (Fig. 4). Then the *K* value can be calculated by dividing the fluorescence intensity of 6,7-dimethoxycoumarin in the upper phase by that in the lower phase (Table 2). Statistical analysis was performed using student's *t*-test, and the results revealed that there is no difference between the *K* value obtained by the two methods ($p = 0.312$).

Table 1
The partition coefficient of components 1 and 2 in n-hexane–ethyl acetate–methanol–water solvent systems.

Number	n-Hexane–ethyl acetate–methanol–water (volume ratio)	Settling time (s)	Volume ratio of two phase (upper/lower)	*K* values	
				Component 1	Component 2
1	3:7:4:9	10	6/23	2.29	2.14
2	1:1:1:1	8	9/11	1.72	1.20
3	1:2:1:1	9	6/5	0.83	1.36
4	1:3:1:1	19	21/10	2.63	2.07

Table 2
K values of component 1 in n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) measured by HPLC and TLC-F method.

Method		Upper phase	Lower phase	*K* values[a]
HPLC (UV, $n=3$)		10,998 ± 130	12,788 ± 298	0.86
TLC-F (I, $n=6$)	Run 1	183	226	0.81
	Run 2	190	241	0.79
	Run 3	180	203	0.88
	Run 4	162	195	0.84
	Run 5	157	187	0.75
	Run 6	171	214	0.92

[a] The differences between the *K* value obtained by the two methods were compared by Student's *t* test and not significant ($p=0.312$).

3.2. Rapid preparation of target compounds using successive sample injection

As can be seen from Fig. 2, n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) is an efficient solvent system for the separation of components 1 and 2. To increase the throughput of separation and reduce the preparation time, a recycling sample injection method was used for rapid separation of the target compounds. As shown in Fig. 5, when the elution of the first sample ends (at 130 min), the second sample injection begins (125 mg of extracts for each injection). The successive separation was completed in 350 min and achieved with satisfactory peak resolution. In total, 35.9 mg component 1 and 66.4 mg component 2 were obtained by this procedure.

In HSCCC, the higher the sample loading, the more asymmetric the peaks of a separation become, causing a loss of resolution and the merging of peaks [20]. However, this problem was solved by using successive sample injection in this study, as shown in Fig. 5, without renewing the column contents, successive sample injections was performed at 130 min, the whole separation was achieved with satisfactory peak resolution and only took 350 min. This suggested that successive sample injection is a useful tool to obtain a large number of target compounds by HSCCC in a short time.

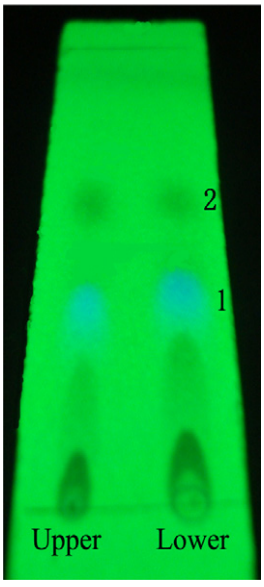

Fig. 3. Separation of PSP extracts by TLC. Mobile phase: chloroform–ethyl acetate (1:1, v/v), 1: 6,7-dimethoxycoumarin, and 2: 5-hydroxymethyl-2-furfural.

3.3. Purity and structure identification of HSCCC peak fractions

The HPLC analyses of the crude extract from PSP and the target compounds are shown in Fig. 6, the purity of the isolated constituents was determined based on the peak area normalized to all observed HPLC peak area. Totally, 42 mg component 1 and 95 mg component 2 were obtained, with the purity of 90.6% and 92.3% respectively (before recrystallization). The NMR data of each component were given as follows: 6,7-dimethoxycoumarin (component 1): ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 3.17 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 6.23 (1H, d, $J=9.3$ Hz, H-3), 6.78 (1H, s, H-5), 7.21 (1H, s, H-8), 7.92 (1H, d, $J=9.3$ Hz, H-4). ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 50.46 (—OCH_3), 57.86 (—OCH_3), 104.62 (C-8), 111.43 (C-6), 112.39 (C-10), 113.53 (C-3), 146.30 (C-4), 147.09 (C-5), 151.36 (C-9), 152.99 (C-2), 162.51 (C-7). Comparing the above data with

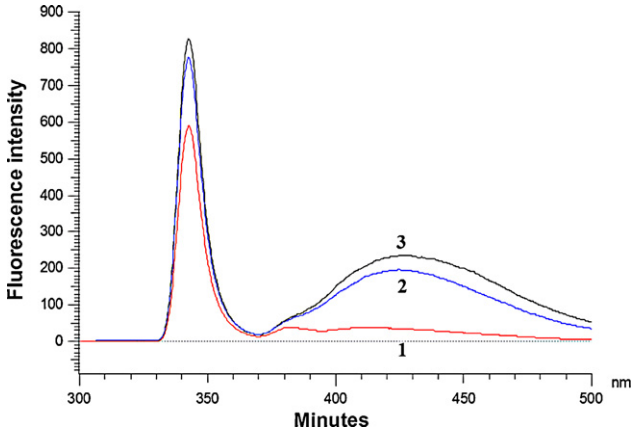

Fig. 4. Fluorescent analysis of the 6,7-dimethoxycoumarin in PSP extracts in upper and lower phase of the n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) after separated by TLC. Excitation: 340 nm, emission: 427 nm; 1: fluorescence spectrum of methanol blank, 2: fluorescence spectrum of upper phase, 3: fluorescence spectrum of lower phase.

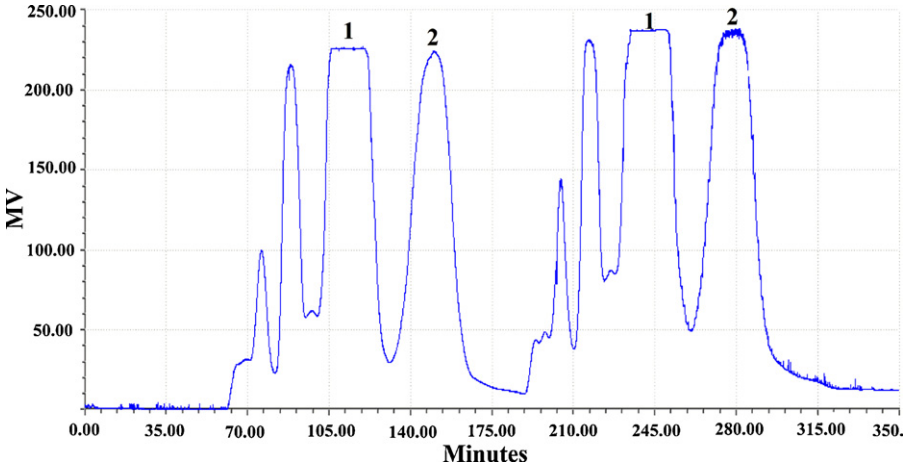

Fig. 5. HSCCC separation of crude extracts from purple sweet potato using successive sample injection. *Conditions*: solvent system: n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v); flow-rate: 2.0 ml/min; rotational speed: 810 rpm; detection wavelength: 254 nm; separation temperature: 25 °C; sample size: 125 mg dissolved in 10 ml upper phase for each injection; retention of the stationary phase: 53%. 1: 6,7-dimethoxycoumarin (collected during 102–124 and 230–254 min); 2: 5-hydroxymethyl-2-furfural (collected during 138–158 and 267–288 min).

the literature [21], the obtained compound was identified as 6,7-dimethoxycoumarin. 5-Hydroxymethyl-2-furfural (component 2): ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.63 (1H, s, —CHO), 7.23 (1H, d, J = 3.3 Hz, H-3), 6.62 (1H, d, J = 3.3 Hz, H-4), 4.64 (2H, s, H-6). ^{13}C NMR (DMSO-d_6, 300 MHz) δ, pm: 179.71 (—CHO), 158.7 (C-5), 154.1 (C-2), 123.4 (C-3), 113.2.7 (C-4), 65.9.2 (C-6). These data were consistent with the literature [22], and component 2 was identified as 5-hydroxymethyl-2-furfural.

The use of thin layer chromatograph coupling with fluorescence spectrophotometry for assay and quality measurement has been reported [23]. In the present study, this method was first applied to select HSCCC solvent systems instead of HPLC method. TLC is cheaper and less complicated than HPLC, provides high sample throughput, and usually requires limited sample pretreatment. While, the fluorometric method is more rapid and sensitive for trace analysis. The TLC-F method is associated with certain advantages. First, this method was more rapid, sensitive and allowed to handle a great number of samples at the same time. Second, after having been separated by TLC, the interference caused by other compounds in extracts could be avoid when measuring the fluorescence intensity of the target compound. Lastly, this method was simple, easy to carry out and possess high accuracy (Table 2). Therefore, the TLC-F method is useful to measure the partition coefficient of target compounds with intrinsic fluorescence such as phenolic compounds, coumarins, alkaloids and flavonoids, etc., in HSCCC solvent systems. Moreover, if the substances possess

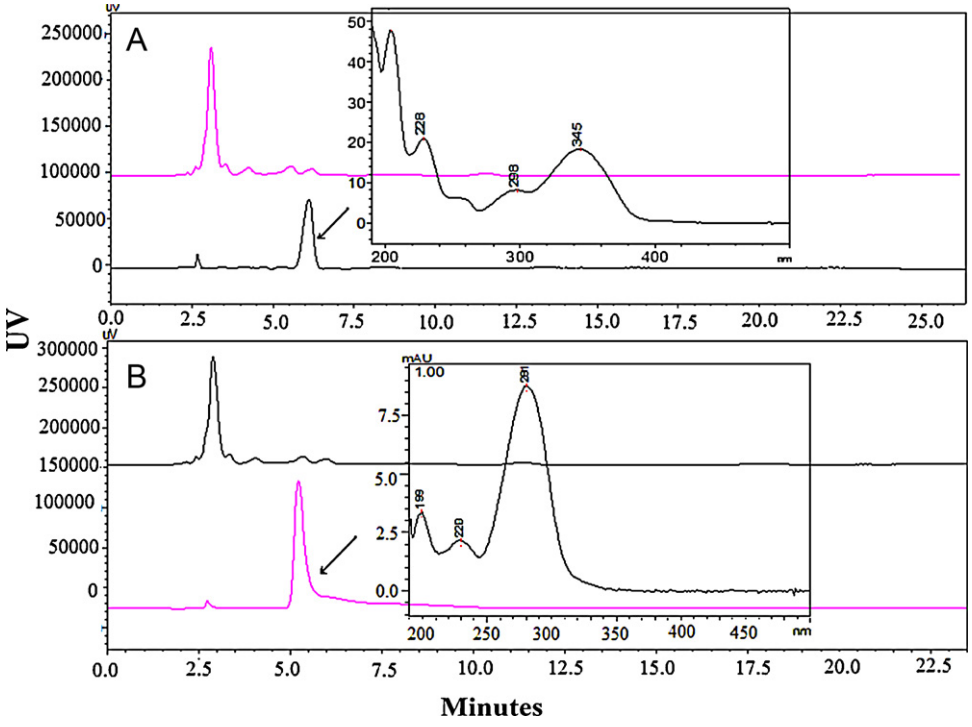

Fig. 6. HPLC analysis of the fraction containing the target compound from silica gel step and the purified components from HSCCC separation. *Experimental conditions*: column: ECOSIL-C18 column (4.6 mm × 250 mm, 5 μm); mobile phase: methanol–water (50:50, v/v); flow-rate: 1.0 ml/min, monitored at 254 nm. A: pre-purified crude sample and 6,7-dimethoxycoumarin; B: pre-purified crude sample and 5-hydroxymethyl-2-furfural; insert: the UV spectrum of corresponding peak.

no fluorescent emission properties (without conjugated electrons), fluorescence quenching and chemical derivatization techniques can also be used for quantitative determination of target compounds [24].

Except for anthocyanins, several bio-active constituents have been identified and isolated from purple sweet potato including chlorogenic acids and caffeic acid esters [25,26]. To the best of our knowledge, this is the first time that 6,7-dimethoxycoumarin and 5-hydroxymethyl-2-furfural have been separated and identified from PSP extracts. In fact, 6,7-dimethoxycoumarin is one of the main active components in *Herba artemisiae scopariae* [27], and has been separated by HSCCC [28]. Previous studies have demonstrated that 6,7-dimethoxycoumarin and its derivatives possess a diverse array of pharmacological and biochemical properties, such as antiangiogenic [29], antibacterial [30], antidiabetic [31], and hepatoprotective effect [32]. Nevertheless, it is important to note that further studies are still needed to evaluate the physiological activity of 6,7-dimethoxycoumarin and 5-hydroxymethyl-2-furfural. Besides, other components in purple sweet potato should be identified and analyzed.

4. Conclusion

In this study, a thin layer chromatograph coupling with fluorometric (TLC-F) method was developed and applied to determine the partition coefficient of target pounds in HSCCC solvent system. And two components were successfully purified by successive sample injection from purple sweet potato for the first time using n-hexane–ethyl acetate–methanol–water (1:2:1:1, v/v/v/v) as the two-phase solvent system. There was no difference between the *K* value obtained by this novel method and by HPLC method. These results suggested that the TLC-F method is useful to measure the partition coefficient of target compounds in HSCCC solvent systems.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.11.040.

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