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Journal of Chromatography A, 1116 (2006) 97–101
JOURNAL OF CHROMATOGRAPHY A
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Application of high-speed counter-current chromatography coupled with high-performance liquid chromatography–diode array detection for the preparative isolation and purification of hyperoside from *Hypericum perforatum* with online purity monitoring

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Received 17 January 2006; received in revised form 1 March 2006; accepted 8 March 2006
Available online 18 April 2006

Abstract

Following preparative isolation and purification by high-speed counter-current chromatography (HSCCC), the collected fractions were generally analyzed by high-performance liquid chromatography (HPLC) to determine the relative purities of each fraction. Our paper reports for the first time a preparative isolation-purity detection hyphenated system: online coupling of HSCCC with high-performance liquid chromatography–diode array detection (HSCCC–HPLC–DAD). The introduction of online purity analysis in HSCCC has dramatically improved the efficiency of this technique by overcoming the drawbacks of post analysis in HSCCC isolation. The effluent from the outlet of HSCCC was splitted into two parts: one was collected, while the other was introduced directly into an HPLC–DAD system for purity analysis through a switch valve. Therefore, the purities of the obtained fractions from HSCCC were monitored, and fractions with high purities were collected. This strategy has been successfully demonstrated with the preparative isolation and purification of hyperoside from *Hypericum perforatum* (*St. Jone's Wort*); a model of TBE-300A HSCCC was used to isolate and separate hyperoside from *H. perforatum* with a two-phase solvent system composed of ethyl acetate–ethanol–water at the volume ratio of 5:1:5 (v/v) using online detection technique. The isolation was done in less than 3.5 h, and a total of 83.0-mg hyperoside at over 99.0% purity was yielded from 300 mg of the partially purified extract. This new strategy possesses general utility in the preparation of bioactive compounds from traditional Chinese medicine (TCM).
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Keywords: Preparative chromatography; Counter-current chromatography; Purity monitoring; *Hypericum perforatum*; Hyperoside

1. Introduction

The purity of compounds is a critical component in the whole process of obtaining the desired biological active material needed for screening and for subsequent formation of structure–activity relationships [1]. Generally, following the purification, the fractions collected on a preparative system are analyzed to determine if the material meets the purity standards required for later-stage screening in lead optimization. With resources expended on collecting and tracking each fraction, it usually is more efficient to collect only the fractions with the desired compounds. Therefore, the great effort in developing and integrating online purity monitoring is one of the major challenging objectives in modern high-throughput purification.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [2], and has been widely used in the preparative separation of natural products [3–5]. UV-triggered purification, owing to its relative simplicity and history of reliability, is widely used in HSCCC. However, with few exceptions [6,7], collecting fractions based on UV response generally results in a large number of samples [8,9]. Therefore, following the preparative isolation and

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doi:10.1016/j.chroma.2006.03.041

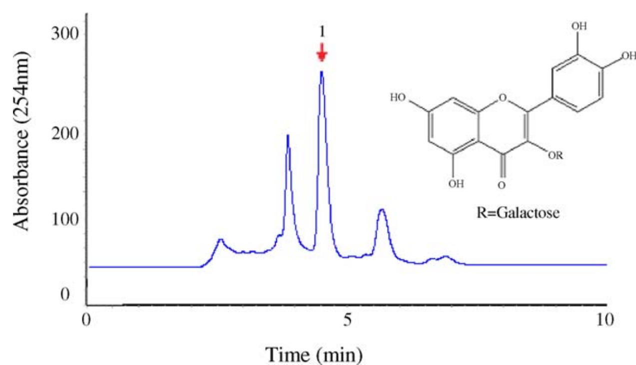

Fig. 1. HPLC chromatogram of the partially purified extract from *Hypericum perforatum*, peak 1: hyperoside. Conditions: column: reversed-phase LiChrospher C_{18} (6.0 mm × 150 mm I.D., 5 μm); mobile phase: acetonitrile–water–acetic acid (25:75:4, v/v); flow rate: 1.0 ml min^{-1}; UV wavelength: 254 nm; column temperature: 30 °C; inject volume: 10 μL.

purification by HSCCC, the fractions collected were generally evaluated by HPLC or TLC combined with densitometry, and on some occasions it may be necessary to dry the fractions and redissolve the residues in an appropriate solvent. These added steps of manual intervention and offline sample processing delay the acquisition of the post-purification sample data and, ultimately, the delivery of compounds for additional testing. Clearly a better solution would be to sample a representative aliquot from each fraction and to automatically analyze the samples. Such technology would streamline the purification/post-purification process by removing these additional steps and decreasing the instrument idle time. In this study, a high-throughput preparative isolation-purity detection hyphenated system has been designed for the first time. It involves online coupling of HSCCC with high-performance liquid chromatography–diode array detection (HSCCC–HPLC–DAD). Online purity monitoring can analyze the representative aliquot from each fraction automatically, and thus, can remove these additional steps and decrease the instrument idle time.

Hyperoside (structure shown in Fig. 1) is the main active component of *Hypericum perforatum* L. (*St. John's Wort*), a perennial herbaceous plant of the Hypericaceae family [10,11]. As a scavenger of reactive oxygen species (ROS), hyperoside has many kinds of biological functions such as inhibiting *E. histolytica* and *G. lamblia* [12], preventing the free radical-induced oxidation of vitamin E in human low-density lipoprotein [13], decreasing the total cholesterol, increasing the superoxide dismutase activity and high-density lipoprotein [14], protecting the apoptosis in rat cardiomyocyte induced by ischemia and reperfusion injury [15], and protecting the gastric mucosal injury in mice induced by ethanol [16]. At present, hyperoside is commercially purified from *H. perforatum* by several steps such as crystallization and chromatography. All these conventional methods are tedious, time consuming, and thus, not suitable for large-scale isolation. To examine the practicability of the new strategy, we have applied HSCCC–HPLC–DAD for the first time to the preparative isolation and purification of hyperoside from *H. perforatum*.

2. Experimental

2.1. HSCCC–HPLC–DAD apparatus

The automated purification system was constructed with a standard Preparative HSCCC system and an online HPLC purity monitoring system. The HSCCC system used for this work consisted of the following commercial components: a model TBE–300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China) with three polytetrafluoroethylene (PTFE) preparative coils (diameter of tube, 2.6 mm total volume, 300 ml), a 20-ml sample loop, a model S constant-flow pump, a model UV-II detector operating at 254 nm, a model N2010 workstation (Zhejiang University, Hangzhou, China), and a 701D fraction collector (Varian) which was used to control fraction collection. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (R) was 5 cm, and the β-value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). Online purity analysis was performed with a six-port, two-position valve including a 10-μL sample loop (C2-2006, Unimicro Technologies, Shanghai, China), a Dionex HPLC system (Dionex, Sunnyvale, CA, USA) including P680 pump, thermostatted column compartment, and PDA-100 photodiode array detector. Chromeleon software (Version 6.50) was used for evaluation and quantification.

Fig. 2 shows the schematic diagram of the hyphenated HSCCC–HPLC–DAD system. The heart of the system is the switch valve and the T-splitter. The six-port, two-position valve (C2-2006, Unimicro Technologies) is used to store the representative aliquot sample from the HSCCC effluent. When triggered, the valve sent a signal to make the collector start to collect the fraction. After 1 min running, the valve was triggered back and began to store the sample for the next run. The T-splitter diverts 1/20 of the flow stream to the switch valve.

2.2. Reagents

D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China).

Ethyl acetate, ethanol, and acetic acid were of analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The *H. perforatum* (*St. John's Wort*) was purchased from a local drug store (Shanghai, China).

2.3. Preparation of the partially purified extract

About 200 g of dried *H. perforatum* was chopped and extracted two times by refluxing with 10,000 ml volume of 70% ethanol in a haven for 2 h. After filtration, the extract was combined and evaporated to dryness by rotary vaporiza-

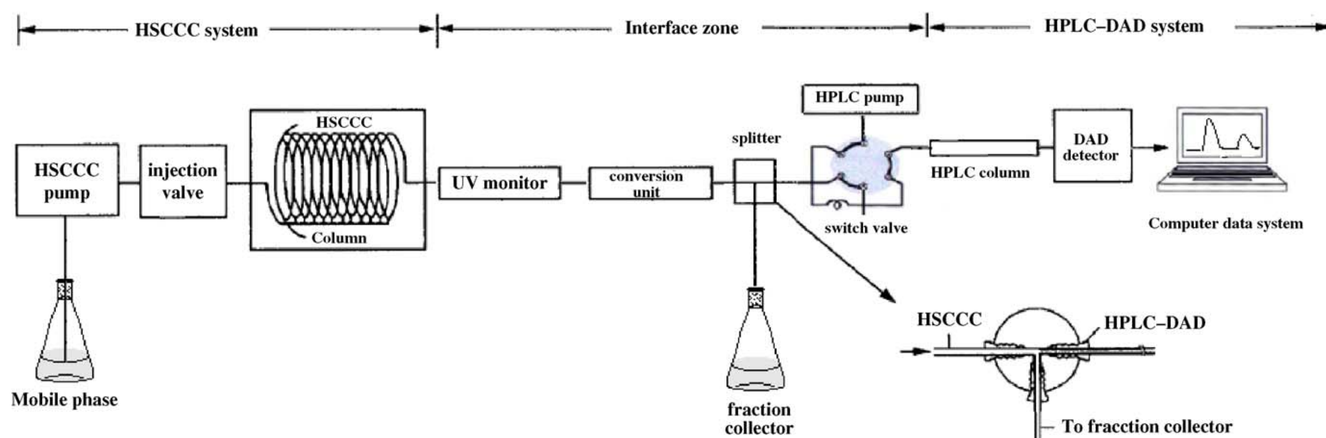

Fig. 2. Schematic diagram of the hyphenated HSCCC–HPLC–DAD system and design of T-split.

tion at 60 °C under reduced pressure, and 800 ml residue was obtained. Half of the residue was re-dissolved in water (total volume 800 ml), which was then chromatographed on a glass column (4.0 cm × 60 cm, contained 350 g D101 macroporous resin). Thousand milliliter of water and 1500 ml of 10% ethanol were first used to elute the resin until the elution was nearly colorless. Then 2500 ml of 40% ethanol was used to elute the target compounds, and 10 elution fractions (250 ml for each) were collected. According to HPLC analysis, five fractions (from 3 to 7) were combined and evaporated to dryness to obtain the partially purified extract, which was subjected to HSCCC for isolation and purification of hyperoside. The sample solution was prepared by dissolving the sample in the 20-ml lower phase of solvent system for isolation and purification. The HPLC chromatogram of the partially purified extract is shown in Fig. 1.

2.4. HSCCC separation procedure

Two-phase solvent system, ethyl acetate–ethanol–water (5:1:5, v/v), was used in the present study. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use. In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system, and then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow-rate of 2.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 20 ml of the sample solution containing 300 mg of the partially purified extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV–vis detector at 254 nm.

2.5. HPLC–DAD purity analysis and identification of CCC peak fractions

When the HSCCC elute time reached 110, 150, 165, 176, 186, 196, 206, and 220 min, the valve was switched on and the factor collector began to collect fractions. In the meantime, the representative aliquot sample was sent to the HPLC–DAD analytical portion and the HPLC–DAD began to perform a purity analysis run. After 1 min, the valve was triggered back for the next analytical run.

The column used was a Lichrospher C_{18} (6.0 mm × 150 mm I.D., 5 μm) (Hanbang Science, Jiangsu, China) with a pre-column equipped with the same stationary phase, the mobile phase was acetonitrile–water–acetic acid (25:75:4, v/v). The solvent flow rate was 1.0 ml min^{-1} and the column temperature was set at 30 °C. The injection volume was 10 μL. The DAD recorded the UV spectra in the range from 190 to 800 nm, and the HPLC chromatogram was monitored at 254 nm.

Identification of the HSCCC pure fraction was carried out by UV (Cary-50, Varian), IR (Hitachi 275-50), MS (Finnigan MAT 711), ^{1}H NMR and ^{13}C NMR (Varian Unity Inova-500).

3. Results and discussion

3.1. Instrumentation

When the PTFE tubing was connected directly to the T-split with two pieces of polyether ether ketone (PEEK) tubing, the relative position of the three outlets had great influence on the effluent due to syphonage in terms of fluid mechanics. To solve this problem, a conversion unit was used to connect the PTFE tubing (1.6 mm I.D.) to a PEEK tubing (0.5 mm I.D.) before the splitter, so that all of the three ports of the splitter are of identical inner diameters. According to the length of the two-outlet PEEK tubing, the 1/20 split ratio was adjusted between the injector and the collector. The conversion unit may probably lead to a backward hydraulic pressure, which would break the two-phase equilibrium in HSCCC system. To test the influence on the resolution and the peak shape, we compared the HSCCC chromatogram obtained using HSCCC–HPLC–DAD system with that obtained using off-line HSCCC system. The results indicated that the backward pressure had no significant influence on the two-phase system.

Generally, the two-phase solvent system used in HSCCC often contains large amount of low-polarity organic solvents such as ethyl acetate and *n*-butanol, which if carried over from the column, tends to cause emulsification in the chromatographic column. This problem can be largely avoided by the T-split

with a small split ratio, through which only 1/20 of the flow stream can be introduced into the HPLC system. In addition, the mobile phase of the HSCCC system is mainly composed of aqueous phase with such minor organic solvent. The results indicated that the tracing of the elution curve had not been disturbed.

3.2. Selection of two-phase solvent system of HSCCC

In HSCCC, a suitable two-phase solvent system was critical for a successful isolation and separation. In our experiment, several kinds of solvent systems including *n*-hexane–ethyl acetate–methanol–water at different volume ratios (10:5:5:6, 5:7:5:6, v/v) and ethyl acetate–ethanol–water at different volume ratios (5:1:10, 5:1:8, 5:1:5, v/v) were tested. After trying the five kinds of solvent systems (Table 1), the solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v) was found to be satisfactory for the separation of hyperoside from the partially purified extract within a short retention time.

3.3. Purification of hyperoside with online purity monitoring by HSCCC–HPLC–DAD

In the present experiment, a total of 83.0 mg hyperoside from 300 mg partially purified extract was obtained in one-step elu-

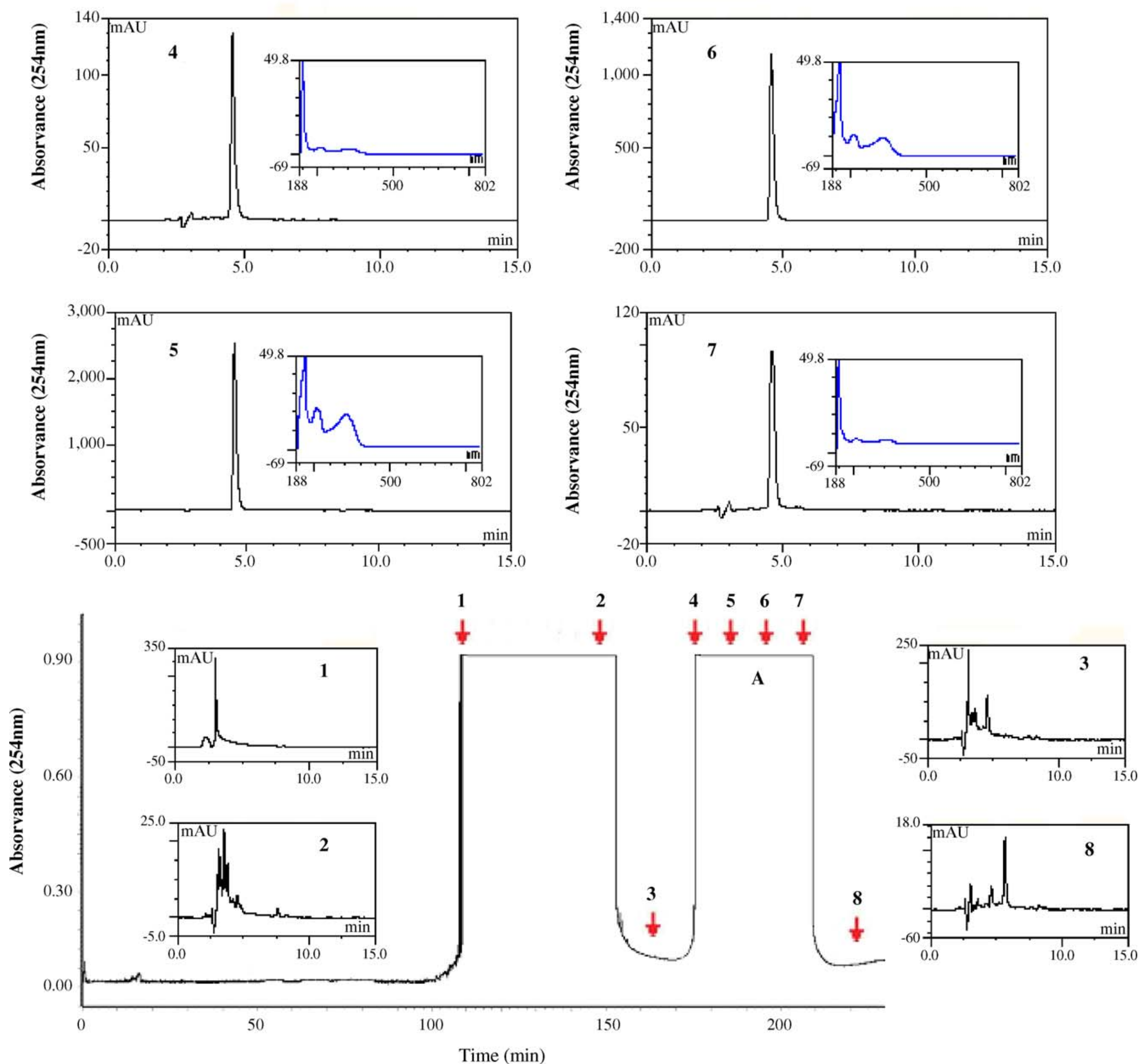

Fig. 3. HSCCC–HPLC–DAD chromatogram of the partially purified extract from *Hypericum perforatum*. Peak A: hyperoside. HSCCC conditions: solvent system: ethyl acetate–ethanol–water (5:1:5, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 ml/min; revolution speed: 800 rpm; separation temperature: 30 °C; sample size: 300 mg; sample loop: 20 ml; detection wavelength: 254 nm. HPLC–DAD analysis conditions are same as shown in Fig. 1. The sample time point: (1) 110 min; (2) 150 min; (3) 165 min; (4) 176 min; (5) 186 min; (6) 196 min; (7) 206 min; (8) 220 min.

Table 1
The K (partition coefficient) values of hyperoside in several solvent systems

No.	Solvent system	K-value
1	n-Hexane–ethyl acetate–methanol–water (10:5:5:6)	6.250
2	n-Hexane–ethyl acetate–methanol–water (5:7:5:6)	3.060
3	Ethyl acetate–ethanol–water (5:1:10)	0.099
4	Ethyl acetate–ethanol–water (5:1:8)	0.246
5	Ethyl acetate–ethanol–water (5:1:5)	0.865

Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 2 mg of partially purified extract placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (K). The K-value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

tion and in less than 3.5 h. Online HPLC purity monitoring of each peak fraction of this HSCCC system revealed that the purity of hyperoside corresponding to peak A was over 99.0%. Fig. 3 shows the HSCCC separation of 300 mg of the partially purified sample with online HPLC chromatogram using the solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v) and the DAD spectrum at different points of peak A.

3.4. Assessment of the applicability of the new technique

In order to assess the applicability of the new technique, a comparison was made between the results obtained using this online sample and analysis technique and the results obtained using traditional post-purification analysis. In order to preserve consistency, 10 μL of each of the off-line evaporated and re-dissolved fractions was analyzed off-line by HPLC–DAD. It can be learnt that the purity results obtained from the online HSCCC–HPLC–DAD agree with those acquired when sampling from fractions that were evaporated and re-dissolved with one of the more traditional methods of post-separation fraction analysis.

3.5. The structural identification

The structural identification of hyperoside was carried out by UV, MS, ^{1}H NMR and ^{13}C NMR spectra as follows: light yellow crystal (MeOH); m.p.: 234–236 °C; UV λ_{max}^{MeOH}: 256, 358, 257, 361 nm. IR (KBR) ν_{max} cm^{-1}: 3300 (OH), 1660 (C=O), 1610, 1510, 1376, 1460 ($C_6H_5^-$). ESI–MS: 465 [M + H]$^+$. ^{1}H NMR (500 MHz, DMSO-d6) δ: 3.50–5.31 (11H), 6.18 (1H, d, J = 2 Hz), 6.38 (1H, d, J = 2 Hz), 6.79 (1H, d, J = 8.5 Hz), 7.50 (1H, d, J = 2 Hz), 7.65 (1H, dd, J = 2 Hz, J = 8.2 Hz), 7.77 (1H, d, J = 8 Hz), 9.08 (1H, brs), 9.65 (1H, brs), 10.78 (1H, brs), 12.70 (1H, brs). ^{13}C NMR (DMSO-d_6) δ: 177.5 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-2), 156.2 (C-8$_a$), 148.4 (C-4′), 144.8 (C-3′), 133.5 (C-3), 121.9 (C-6′), 121.1 (C-1′), 116.0 (C-5′), 115.2 (C-2′), 103.9 (C-4a), 101.7 (C-1″), 98.7 (C-6), 93.6 (C-8), 75.8 (C-5″), 73.3 (C-3″), 71.3 (C-2″), 67.9 (C-4″), 60.1 (C-6″).

4. Conclusion

A novel preparative isolation-purity detection hyphenated system, HSCCC–HPLC–DAD, was established for the first time, and the online purity monitoring has improved the efficiency of the overall purification process. This strategy has been successfully demonstrated with the preparative isolation and purification of hyperoside from *H. perforatum* by HSCCC–HPLC–DAD with a two-phase solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v). The isolation was done in less than 3.5 h, and the purity of the obtained hyperoside was over 99.0%.

The result shows that the purity results obtained from the online HSCCC–HPLC–DAD agree with those acquired when sampling from fractions that were evaporated and re-dissolved with one of the more traditional methods of post-separation fraction analysis. It is envisioned that this approach could be adopted as a new technical platform for a wide range of different HSCCC–HPLC–DAD automated purification system to isolate chemical constituents from TCM, in general.

Acknowledgement

This work was supported by the Fundamental Research Key Project founded by the Science and Technology Department of Shanghai, China, Grant no. 03JC14005.

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