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Short communication

Comparison of high-speed counter-current chromatography instruments for the separation of the extracts of the seeds of *Oroxylum indicum*

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Abstract

Analytical Milli high-speed counter-current chromatography (HSCCC) was used for the selection and optimization of the two-phase solvent system to separate flavonoids from the extracts of the seeds of *Oroxylum indicum*. The optimum solvent system obtained from Milli-CCC was also the best solvent system for preparative HSCCC and led to the successful separation of two crude flavonoids from the seeds of *O. indicum* by Lab/Prep (laboratory preparative) HSCCC using different sized coils. Four flavonoids were isolated by preparative HSCCC: baicalein-7-O-diglucoside (25.0 mg, 92% purity), baicalein-7-o-glucoside (50.4 mg; 95% purity), baicalein (75 mg; purity 98%) and chrysin (100 mg; purity 98%).

Keywords: *Oroxylum indicum*; High-speed counter-current chromatography; Flavonoids

1. Introduction

The selection of suitable solvent systems is the first and most important step in performing preparative HSCCC. A test-tube solvent system shaking test, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are used to choose suitable solvent systems for HSCCC. To a certain extent, TLC provided useful information for the solvent selection process. But sometimes, TLC gave false information due to the use of a solid support. Several papers have already shown that analytical HSCCC is very useful for rapid solvent system selection and method development of preparative HSCCC and may become a useful method for the following applications: (1) in the development of methods for preparative HSCCC, (2) microscale separations, and (3) measurements of partition coefficients for preparative HSCCC [1,2]. Indeed, analytical HSCCC is already supporting method development and solvent selection in preparative HSCCC with its short separation timespeedy and minimum solvent consumption [3].

Oroxylum indicum is a small to medium sized tree found in China and India and its importance has been described in a previous paper [4]. Flavones [4,5] sterols and prunetin [6] have been reported in different parts of the tree. In the seeds of *O. indicum*, amounts of bioactive flavonoids such as baicalein-7-O-diglucoside, baicalein-7-O-glucoside, baicalin, chrysin, apigeninhave been identified [7]. In recent studies, baicalein and chrysin have been reported to show anti-inflammatory, anti-allergic [8] antioxidant and anticancer activities and baicalein showed a hypertensive effect [9,10]. A recent clinical test has identified that the extracts of its seeds can result in body weight loss and reduce postprandial blood–glucose. In this paper, analytical HSCCC is used for the systematic selection and optimization of the two-phase solvent system for the separation of flavonoids of the

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ethyl acetate extracts from the seeds of *O. indicum*. Using the optimized solvent system, preparative HSCCC separation of the crude flavonoids are performed from the crude extract of the seeds of *O. indicum* on three different sized coils.

2. Experimental

2.1. Solvent and reagents

Analytical grade solvents used in this study included hexane, ethyl acetate, methanol and water. All these solvents were purchased from Fisher Chemicals (Loughborough, UK). All solvents were degassed prior to use.

2.2. Apparatus

Two HSCCC instruments were used in our research. Both were supplied by Brunei Institute for Bioengineering, Brunei University, Uxbridge, UB8 3PH, UK. One is a new low volume capacity Milli-CCC device, achieving high resolutions in minutes as opposed to hours [11]. The Milli-CCC J-type centrifuge has gears enclosed in a lubricated case to minimize noise. Its volume with one coil mounted in a cantilever style is 4.6 ml with 10 m of 0.76 mm bore tubing. The extra coil volume is 0.34 ml and (three range 0.68–0.79. It was run at its maximum speed of 2100 rpm. The other one is a Lab/Prep J-type coil planet centrifuge HSCCC. This CCC has four coils of 1.6 mm bore PTFE tubing that are wound tightly on two separate bobbins on one rotor; each bobbin containing two concentrically wound coils of PTFE tubing with a total volume of 495 ml. In our studies, a rotational speed of 1100 rpm was used and three column configurations were chosen. Their column volumes with (β values) were 49.9 ml (0.83–0.86), 94.9 ml (0.80–0.86) and 169.9 ml (0.70–0.80).

2.3. Sample preparation

The seeds of *O. indicum* were ground into powder after freezing them by liquid nitrogen. Two hundred and fifty grams of a yellow powder of seeds was refluxed for 8 h in 1000 ml of 90% methanol; the extract was then filtered and evaporated. The residue was redissolved in 500 ml water and extracted three times with ethyl acetate. The layer of ethyl acetate was evaporated to dryness and yielded 25.5 g of a yellow powder. The layer of water was extracted with 500 × 3 ml butanol and the layer of butanol was evaporated to dryness and yielded 15.5 g of a deep yellow powder. The extract from ethyl acetate was labelled as sample 1 and the extract from butanol was labelled as sample 2.

2.4. HSCCC separation procedure

A biphasic mixture of hexane–ethyl acetate–methanol–water was prepared in different ratios and purged 10 min with nitrogen to remove any dissolved gases. First, the coil was filled with the upper phase (organic layer: hexane–ethyl acetate) of the biphasic mixture. The coils were rotated in a direction to orient the coils head (centre) to tail (periphery) and at the speeds given in figure legends. Lower phase (aqueous: methanol–water) was pumped into the coil from head to tail at a constant flow rate. At first stationary (upper phase) was displaced. When the mobile phase came through and two layers were observed, the equilibration point was determinate when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be calculated by subtracting the volume of the stationary phase eluted at the end of the equilibration process and extra volume from the total volume [12].

2.5. Analyses and structural identification of CCC peak fractions

All peak fractions were analyzed using on-line high-performance liquid chromatography–mass spectrometry (LC–MS) and their retention times compared to those from standard materials.

3. Results and discussion

3.1. Separation of flavonoids of sample 1 by Milli-CCC

During the selection of two-phase solvent systems, solvent systems composed of chloroform–methanol–water and hexane–ethyl acetate–methanol–water with different volume ratios were applied for sample separation. Both solvent systems can affect the separation of crude flavonoids but a solvent system composed of hexane–ethyl acetate–methanol–water was chosen for the separation of crude flavonoids for environmental considerations. When a solvent system composed of hexane–ethyl acetate methanol–water (1:1.2:1:1) was applied to the separation of crude flavonoids from the seeds of *O. indicum*, Milli-CCC gave good resolution for the separation of flavonoid components of sample 1. Three components 2–4 were resolved very well and component 1 was retained in the stationary phase and was pumped out afterwards. Fig. 1 shows the separation results at different flow rates by Milli-CCC. Sf was defined as the percentage of stationary volume retained in the coil divided by the coil volume and the $k = 1$ point (the system volume) was marked by the arrow.

Fig. 1 shows that Milli-CCC separates three components in 25 min at flow rate of 0.5 ml/min and gives very good resolution for the separation of sample 1. When increasing the flow rate to 2.0 ml/min, three components can be separated in only 6 min still with good resolution. The whole consumption of solvent is only 30 ml.

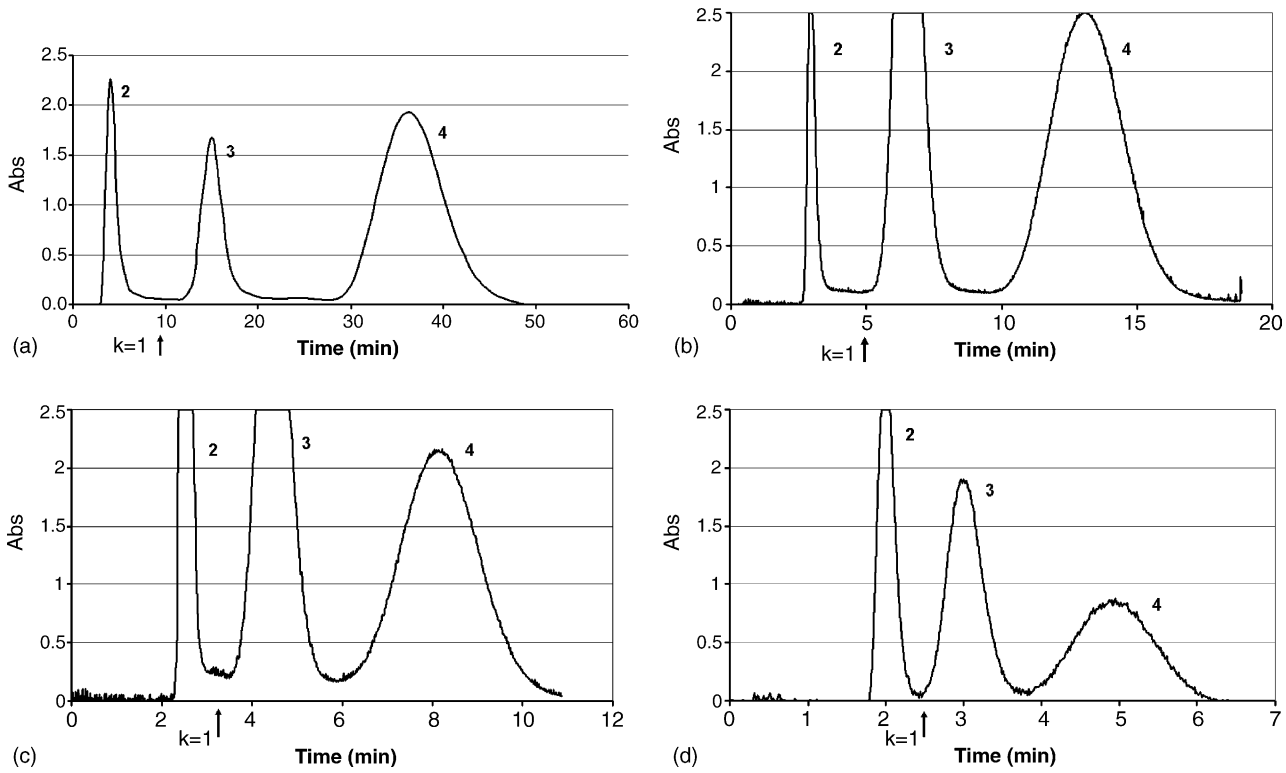

Fig. 1. HSCCC chromatogram of the separation of sample 1 at different flow rates by Milli-CCC: (a) 0.5 ml/min, Sf = 75.2%; (b) 1 ml/min, Sf = 50.4%; (c) 1.5 ml/min, Sf = 33.2%; and (d) 2.0 ml/min, Sf = 30.3%. *Experimental conditions:* coil volume: 4.6 ml; solvent system: hexane–ethyl acetate–methanol–water (1:1.2:1:1); stationary phase: organic phase; mobile phase: aqueous phase; rotation speed: 2100 rpm; sample volume: 0.2 ml; sample concentration: 20 mg/ml; direction of motor: forward direction.

3.2. Separation of flavonoids of sample 1 in column volume of 49.9 ml

When the same solvent system composed of hexane–ethyl acetate methanol–water (1.2:1:1) was employed for the separation of sample 1 using HSCCC with the smallest 49.9 ml coil, similar results were obtained (see Fig. 2) three components were separated in 100 min with good resolution. Fractions were collected from 34 to 43 ml (peak 2), 50 to 63 ml (peak 3) and 73 to 95 ml (peak 4) and pumpout (peak 1) and evaporated to dryness, analysed by HPLC and found to be more than 98% pure. This result shows that the optimized separation conditions of Milli-CCC can be used to evaluate solvent system for semi-preparative HSCCC.

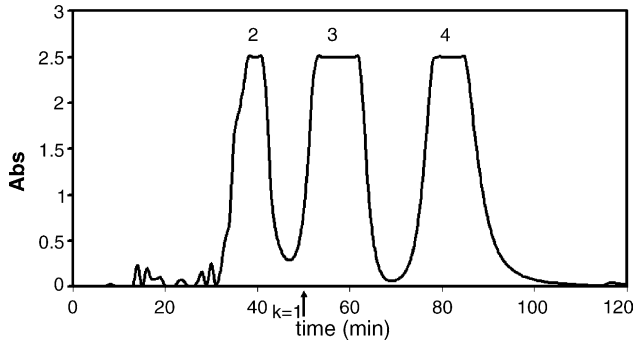

Fig. 2. Chromatogram of the separation of sample 1 using the analytical/preparative HSCCC. *Experimental conditions*: coil volume: 49.9 ml; stationary phase: organic phase; mobile phase: aqueous phase; rotation speed: 1100 rpm; flow rate: 1.0 ml/min; sample volume: 1.0 ml; sample concentration: 20 mg/ml; solvent system: hexane–ethyl acetate–methanol–water (1:1.2:1:1); direction of motor: forward direction; Sf = 41.8%.

3.3. Separation of flavonoids of sample 2 by Milli-CCC

Sample 2 is also the crude flavonoids of butanol extracts from the seeds of *O. indicum.* When a solvent system of hexane-ethyl acetate–methanol–water (1:8:1:8) was applied to the separation of the crude flavonoids of sample 2, a good resolution was obtained. Two components were isolated and labelled as components A and B. Fig. 3 shows the separation of sample 2 by Milli-CCC.

In this Milli-CCC, the whole separation time can be finished in 14 min with good resolution of components A and B and very little solvent being consumed in the process. Components A and B were, respectively, identified as baicalein-7-O-diglucoside and baicalein-7-O-glucoside by comparing their HPLC retention time with those of standard materials. Component A gave the same retention time as that of peak 1 of sample 1 and component B gave the same retention time as that of peak 2 of sample 2. When the above solvent system composed of hexane–ethyl acetate–methanol–water (1:8:1:8)

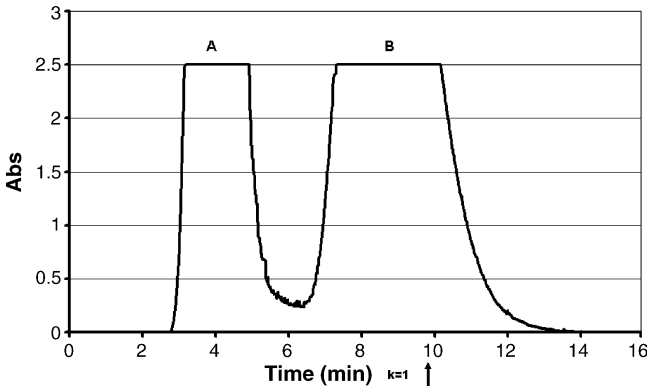

Fig. 3. HSCCC chromatogram of the separation of sample 2 by Milli-CCC. *Experimental conditions*: coil volume: 4.6 ml; stationary phase: organic phase; mobile phase: aqueous phase; rotation speed: 2100 rpm; solvent system: hexane–ethyl acetate–methanol–water (1:8:1:8); flow rate: 0.5 ml/min; sample volume: 200 μl; sample concentration: 20 mg/ml; direction of motor: forward direction; Sf = 80.9%. $K = 1$ (with arrow) represents the theoretical elution time of the coil volume plus dead volume.

was used with the preparative HSCCC for the separation of sample 2 in column volumes of 49.9, 94.9 and 169.9 ml, the results are shown in Fig. 4.

3.4. Comparison of partition coefficients

Our studies of different scale HSCCC instruments from analytical (4.6 ml) to semi-preparative (49.9 ml) HSCCC for sample 1 show that Milli-CCC separations at 2 ml/min (Fig. 1d) give very good prediction of phase partition behaviour at the higher scale (Fig. 2). The partition coefficient values measured from the chromatograms were 0.3, 1.8 and 4.5 for Milli-CCC with peak resolutions Rs_{12}, Rs_{23} and Rs_{13} values of 1.56, 1.32 and 2.37, respectively, and for the Brunel-CCC were 0.4, 1.3 and 2.4 with peak resolution values of 1.29, 1.51 and 3.00, respectively. Our studies of different HSCCC instruments from analytical CCC (4.6 ml) to preparative CCC (169.9 ml) for the separation of sample 2 showed that the solvent system and optimum conditions of analytical CCC give the same results as those of preparative HSCCC as it is quick and the solvent consumption is low. For this phase system, the Milli-CCC separation at 0.5 ml/min was a good predictor for phase partition behaviour at the higher scale. Their partition coefficients for Milli-CCC (4.6 ml at 0.5 ml/min) were calculated from the chromatograms as $K_A = 0.22$ and $K_B = 0.85$ with a resolution $Rs = 1.36$ compared to the larger coil on the Brunei CCC (170 ml at 2.0 ml/min) with a $K_A = 0.08$ and $K_B = 0.58$ and a resolution of 0.90.

The solvent system of hexane–ethyl acetate–methanol–water is a good choice for separation of flavonoids from natural products by HSCCC. Note that the partition coefficient (K) was defined as the solute concentration in the aqueous (stationary) phase divided by that in the organic (mobile) phase.

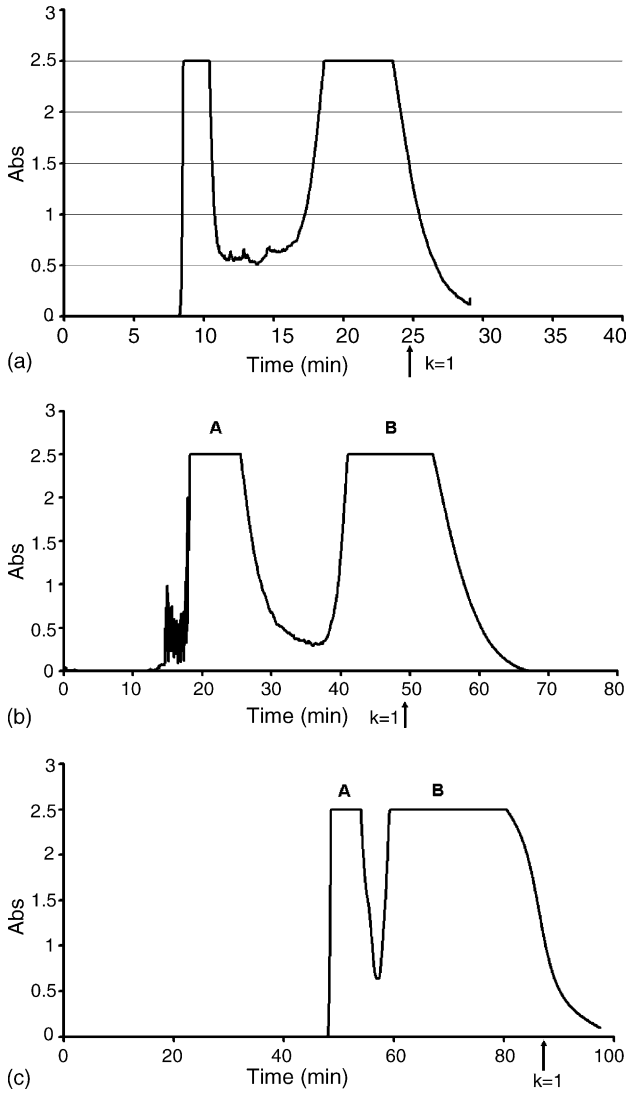

Fig. 4. HSCCC chromatograms of the separation of sample 2 on the preparative HSCCC with different volume of coils. *Experimental conditions*: solvent system: hexane–ethyl acetate–methanol–water (1:1.2:1:1); stationary phase: organic phase; mobile phase: aqueous phase; rotation speed: 800 rpm; sample concentration: 20 mg/ml; flow rate: 2.0 ml/min; direction of motor: forward direction; coil volumes: (a) column volume 49.9 ml, Sf = 72.1%, sample volume, 1.0 ml; (b) column volume 94.9 ml, Sf = 65.3%, sample volume, 2.0 ml; (c) column volume 169.9 ml, Sf = 44.9%, sample volume, 4.0 ml.

4. Conclusion

We compared different column volumes from analytical to preparative scale for the separation of flavonoids from the seeds of *O. indicum*. Analytical CCC gave similar resolution to preparative CCC. Hence, analytical CCC is the best way for rapid selection of solvent system for preparative CCC. In Milli-CCC, the whole consumption of solvent from run to run is a few millilitres in minutes as opposed to hours. This separation time can compete with that of HPLC. Analytical CCC therefore is a valuable complementary analytical tool in analytical laboratories.

References

[1] F.Q. Yang, T.Y. Zhang, R. Zhang, Y. Ito, J. Chromatogr A 829 (1998) 137.
[2] T.Y. Zhang, X. Hua, R. Xiao, S. Knog, J. Liq. Chromatogr. 11 (1988) 233.
[3] D. Fisher. I.J. Garrard. R. Van Den Heuvel., F.E. Chou, J.W. Fahev, I.A. Sutherland, J. Liq. Chromatogr. Rel. Tech., in press.
[4] L.J. Chen, D.E. Games, J. Jones, J. Chromatogr. A 988 (2003) 95.
[5] T.T. Suyoshi, I. Maro, K. Haruhisa, N. Tsuneo, Shoyak Gaku Zasshi (1988) 41.
[6] K.C. Joshi, L. Prakash, S.P. Sapra, Curr. Sci. 58 (1989) 929.
[7] T. Tomimori, Y. Imoto, M. Ishida, Shoyakugaku Zasshi 42 (1) (1988) 98.
[8] S. Ikemoto, K. Sugimura, N. Yoshida, Cancer Lett. 160 (2000) 219.
[9] R. Kyo, N. Nakahata, I. Sakakibara, M. Kubo, Y. Ohizumi, J. Pharm. Pharmacol. 50 (1998) 1179.
[10] H. Takizawa, A.M. DelliPizzi, A. Nasjletti, Hypertension 31 (1998) 866.
[11] L. Janaway, D. Hawes, S.N. Ignatova, P. Wood, I.A. Sutherland, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 1345.
[12] P.L. Wood, D. Hawes, L. Janaway, Ian A. Sutherland, J. Liq. Chromatogr. Rel. Technol. 26 (9 and 10) (2003) 1373.